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James M. Krueger

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Department of Physiology and Biophysics
University of Tennessee, Memphis
894 Union Avenue
Memphis, Tennessee 38163

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The broad objective of this USAMRDC-supported research is to develop the information needed to ascetain whether muramyl peptides (MPs) and/or other endogenous sleep factors (SFs) may be useful as somnogenic agents. This concern originated from our previous research in which we described MPs, isolated from brain and urine, as somnogenic agents. Subsequently our results were confirmed and we and others greatly expanded upon those observations. Several additional SFs have been identified that may be involved in the cascade of biomedical events involved in regulation of sleep, e.g. interleukin-1 (IL1). Thus, we think it possible that new more effective somnogenic agents could be developed using MPs and/or other putative SFs. In the second year of this contract six experiments were performed and a summary of results from each are as follows:

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1) Additional MP structure - MP somnogenic activity relationships were determined. We found that 6-O-acetylation of the muramic acid moiety enhanced somnogenic activity and that MP dimers, whether formed by peptide or glycosidic linkage, were relatively inactive as somnogenic agents.

2) The preoptic area (POA) of the brain has been implicated in sleep regulation. It was of interest, therefore, to determine whether animals with a lesioned POA could still respond to a MP. We found that the direction and magnitude of MP-induced sleep responses were similar before and after POA-lesions although the baseline values upon which the changes were superimposed had shifted.

3) Relationships between sleep and infectious disease have never been examined systematically. Our interest in this relationship resulted from the close structural homologies between the somnogenic MP we isolated and the MP-monomeric building blocks of bacterial peptidoglycan. To address this issue, the effects of Staphylococcus aureus on rabbit sleep were determined. Briefly, sleep patterns were greatly altered over the course of the infections.

4) Endotoxin and its lipid A moiety are components of gram-negative bacteria that share with MPs the ability to induce increased IL1 production. Since IL1 is postulated to be involved in MP- and other SF-induced sleep it was of interest to begin to investigate lipid A structure - lipid A somnogenic activity relationships. We found that the number, structure and position of acyl residues on the disaccharide backbone of lipid A molecules, as well as the number of phosphate groups, influence their somnogenic activities.

5) Alpha-melanocyte-stimulating hormone (aMSH), a proopiomelanocortin-derived peptide which acts as a physiological inhibitor of some of IL1 actions. It was, therefore, of interest to determine the somnogenic actions of aMSH. We found that aMSH inhibited normal sleep and also antagonized IL1-enhanced sleep.

6) Several lines of evidence suggest that substances involved in the regulation of growth hormone (GH) secretion are also involved in sleep regulation. For example, IL1 induces GH release, possibly via a step involving GH-releasing factor (GRF). Thus, we tested GRF for somnogenic activity. We found that GRF enhanced both slow-wave-sleep and rapid-eye-movement sleep in rats and rabbits.

The above results are discussed within the context of sleep-immune interactions and a model we developed this year relating the somnogenic actions of various SFs.

FOREWORD

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

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INTRODUCTION

The postulate that a sleep-promoting substance(s) (sleep factor: SF) accumulates in brain during wakefulness had its origin in the ancient observation that prolonged wakefulness results in a strong propensity for sleep. Early in the twentieth century, the groups of Ishimori and Pieron demonstrated the presence of sleep-promoting substances in cerebrospinal fluid. Over the next 50 years, several attempts to repeat their experiments were successful (reviewed 89). Within the past few years, there have been intense efforts to characterize SFs; these studies have been stimulated by the expectations that such investigations would help elucidate the functions and brain mechanisms of sleep as well as provide new and safer somnogenic agents. Indeed, a variety of substances have recently been proposed as SFs (reviewed 90).

Our efforts to isolate and identify SFs began about 15 years ago when a sleep-promoting substance, factor S, was first described as a constituent of cerebrospinal fluid (CSF) that increases in concentration during sleep deprivation (121). Subsequently, we identified somnogenic substances derived from brain and urine as muramyl peptides (MPs); the most active component isolated is N-acetyl glucosaminyl-1,6-anhydro-N-acetyl-muramyl-alanyl-glutamyl-diaminopimelyl-alanine (NAG-1,6 anhydro-NAM-ala-glu-dap-ala) (100). One picomole of this substance given to rabbits is sufficient to enhance slow wave sleep (SWS) for 6 or more hours. This substance and several chemical analogs to it were synthesized or obtained from bacterial peptidoglycan and tested for somnogenic activity. Specific structural requirements for somnogenic activity were identified (reviewed 81, and see Results).

Certain MPs are also immune response modifiers (31) and pyrogenic (129). MPs alter cytokine production (e.g., interleukin [IL1] [31]) and prostaglandin (PG) metabolism (reviewed 82). Indeed, these substances are postulated to be mediators of the biological activities of MPs, in that certain cytokines (e.g., IL1 [73], tumor necrosis factor [TNF] [85], and interferon α [INFα] [86]) and PGs (e.g., PGD_2 [59, 82]) are somnogenic, alter body temperature and affect certain aspects of the immune response. Such consideration led us to propose that a cascade of biochemical events, involving, in part, MP induction of IL1 production and IL1 induction of PG synthesis, is involved in sleep regulation (90). More recently, we have expanded this proposal and presented a scheme for sleep regulation that shows the interrelationship between most of the putative SFs (89). Thus, within the past two years, the number of putative SFs has expanded, and a postulate concerning their interaction with regard to sleep regulation has been provided.

At the start of our contract period (June 1, 1986), our evidence suggested that MPs have the capacity to enhance SWS. Several laboratories had confirmed those findings, and we and others provided evidence suggesting that the pyrogenic and immunologic activities of MPs could be separated from their somnogenic actions. Because of these considerations, we thought it likely that in the long run, new, more effective and safer somnogenic agents could be developed using MPs and/or other substances involved in the cascade of biochemical events involved in regulation of normal sleep. Therefore, the broad objective of our studies is to develop the information needed to ascertain if it is reasonable to propose MPs or other endogenous SFs as potential somnogenic agents. In the first year of our contract period, five sets of experiments were performed with this goal in mind; specific results are described in our

first annual report (dated June 1, 1987). With the same objective in mind, this year we performed six sets of experiments. In this report, these experiments are numbered one through six as follows: 1) We began by expanding our knowledge concerning MP structures/MP somnogenic activity relationships. 2) In our second set of experiments we sought to determine whether an intact preoptic area was necessary for MP-induced sleep and temperature responses. 3) Next, because of the known structural relationships between MPs and bacterial cell wall peptidoglycan, we asked the question whether sleep was altered during bacterial infection. We also view this work as very important from a practical point of view. 4) We determined several lipid A structures/lipid A somnogenic activity relationships. 5) We expanded our knowledge concerning IL1-enhanced sleep by investigating the effects of a known IL1 inhibitor, αMSH, on IL1-enhanced sleep. 6) Finally, we investigated the somnogenic actions of growth hormone releasing factor.

METHODS

I. Sleep bioassay

A. Animals and surgery. Adult male New Zealand White Pasteurella-free rabbits, weighing 3-4 kg, were obtained from Myrtle's Rabbitry (Thompson Station, TN). Under ketamine-xylazine anesthesia (35.0-5.0 mg/kg, subcutaneously), animals were provided with chronically implanted electroencephalographic (EEG) electrodes, a glass bead thermistor, and a cerebral ventricular guide tube as previously described (83). Stainless steel screws were implanted over the frontal, parietal, and occipital cortex. In most animals, a 1-mm diameter 50-kΩ thermistor (Fenwall Electronics, No. GA45J1) was placed through

a burr hole 4 mm lateral and 1 mm posterior to bregma, 1-2 mm into the parietal cortex. Wires from Amphenol plugs (No. 223-1509) were soldered to the screws and to the thermistors for EEG and brain temperature (T_{br}) recordings. A cerebral ventricular guide tube was also implanted 4 mm lateral and 1 mm posterior to bregma on the side opposite that of the thermistor. During implantation, pressure at the tip of the infusion needle was monitored and used as a guide to ensure that the needle was in a lateral ventricle as previously described (83). Dental acrylic (DuzAll®) was used to secure the guide tube, thermistor, and EEG plugs to the skull; it also served to insulate the leads. After the operation, a topical antibiotic (Bacitracin®, Lilly) was applied to the incision, and 5 mg/kg of gentamicin sulfate was injected intramuscularly. At least one week was allowed for recovery.

B. Experimental procedure. For intracerebroventricular (ICV) injections, appropriate amounts of the test substances were diluted to 25 µl with phosphate-buffered saline (PFS) or artificial CSF (77); these solutions were slowly injected into a lateral ventricle over a 5-min period. For intravenous (IV) injections, appropriate amounts of the test solutions were diluted to 0.5 ml in PFS and then rapidly injected into a marginal ear vein. In most cases, to control for injection effects, rabbits also received IV or ICV injections of PFS or artificial CSF. In addition, in all cases, recordings were obtained from each rabbit without treatment on days separated from experimental days by at least one week. A within-subject experimental design could thus be used because neither IV nor ICV injections of PFS or artificial CSF affected the parameters measured. Individual rabbits that received repeated injections were not used more than once every 10 days. Under these conditions, signs of tolerance were not observed.

Animals were housed in rooms maintained at 21 ± 2°C on a 12:12 h light: dark cycle (0600-1800 h light). Animals were brought to the experimental cages (Hotpack, model 352600) the day before an experiment for an overnight acclimation period. These chambers were also kept at 21 ± 2°C on a 12:12 h light:dark cycle. Food and water were available ad libitum at all times. A cable from the animals was connected to a BRS/LVE electrical contact swivel, allowing the rabbits to move freely during recording periods. The other end of the swivel was connected via a cable to a Grass polygraph (model 7D). Rabbits were connected to the recording cable for a 1-h habituation period before each recording period; data were not collected during this time. Animals were then taken out of the experimental cage for about 10 min and were given the test substance. Before and after injection, colonic temperatures (T_{co}s) were determined using precalibrated thermistor probes (Yellow Springs Instruments, #49TA) inserted 10 cm into the colon. Rabbits were then returned to the experimental cages for a 6-h recording period. T_{co}s were again determined after the recording period. Injections took place between 0800 and 1000 h. ICV injection doses are expressed in grams because adult rabbit brains weigh about 8 g, regardless of body weight. Any variations in this protocol are described below under the specific experimental protocols.

C. Sleep and temperature measurements. To define states of vigilance (see below), the EEG, ratios of theta/delta cortical EEG activity, T_{br}, and body movements were recorded. The cortical EEG was led to a Buxco (Sharon, CT) DL-24 EEG analyzer; the rectified average voltages in the 0.5-4.0 Hz (delta), 4-8 Hz (theta), 8-12 Hz (alpha), and 12-25 Hz (beta) frequency bands were printed on paper each minute. The ratios of theta/delta voltages were also computed, and these values were continuously recorded simultaneously with

the EEG on polygraph paper. The Buxco and Grass amplifiers, filters, and averagers were calibrated using sine waves of known frequency and peak-to-peak voltages. To determine T_{br}, implanted thermistors were calibrated relative to T_{co} as previously described (78). This method is based on the observation that T_{br} follows T_{co} during fever. Rabbit body movements were monitored using a Grass tremor transducer (model SPAI) attached to the recording cable.

Periods of wakefulness (W), SWS, and REMS were determined by visual analyses of polygraph recordings. Recordings were divided into 12-sec epochs, and each epoch was classified as either W, SWS, or REMS using the following criteria. W was associated with low-voltage EEG, body movements, midlevel range of theta/delta ratios, and a decreasing T_{br} after REMS episodes or increasing T_{br} after SWS episodes. SWS was characterized by high EEG slow-wave voltage, little or no body movement, low theta/delta ratios, and a decreasing T_{br}. REMS was identified by a low-voltage EEG, phasic body movements, high theta/delta ratios, and a relatively rapid increase in T_{br}.

The percentages of time spent in SWS and REMS were determined for each hour and for the total recording period. In some cases, printed average voltages were used to calculate hourly mean voltage in each frequency band. Average voltages were also used to determine maximum delta-wave (0.5-4.0 Hz) values during 1-min periods. For these determinations, the 10 highest 1-min values that occurred during the 6-h recording period were averaged for each rabbit. For each value used, the EEG was checked to ensure that such values were not associated with EEG artifact; all values used occurred during SWS

episodes. Student's t-tests for paired data were used for comparison between data obtained from the same animal under experimental and control conditions. A significance level of $p < 0.05$ was used. Other statistical procedures used are described below under specific experimental protocols.

II. Specific Experimental Protocols

A. Experiment No. 1: MP structure/MP somnogenic activity relationships

This year, 19 new MPs were assayed for somnogenic activity using the procedures described above. Each substance was tested in 4 or more animals and in most cases, 2-3 doses of each substance were tested. Substances 1-6 and 9-11 (Table 1) were isolated and purified from Neisseria gonorrhoeae as described previously (87) and in last year's annual report. Several peptidoglycan dimers (substances 7, 8, and 12-15; Table 1) were derived from Actinomadura sp. strain, strain R39, and were a gift from M. Guinard of the University of Claude Bernard, Villeurbanne, France. In addition, four synthetic MPs were tested (substances 16-19; Table 1). These synthetic substances were gifts from V. Kovalzon, Moscow University, USSR. Substances 1-15 were purified and their structures were verified using mass spectroscopy as previously described (100).

B. Experiment No. 2: Effects of preoptic area lesions on MDP-induced sleep and fever responses

1. Animals and Surgery. Twenty-two male New Zealand albino rabbits (Myrtle's Rabbitry, Thompson Station, TN), weighing from 3.0-5.0 kg,

were used in this study. General anesthesia was induced by the subcutaneous administration of 35 mg ketamine/kg and 5 mg xylazine/kg. Additionally, Xylocaine® (Astra, NY) was injected subcutaneously in the scalp where the skin was to be incised. Using stereotaxic guidance (T. H. Wells, Jr., Mechanical Developments Co., South Gate, CA) and the atlas of Urban and Richland (165), bregma and lambda were leveled in the horizontal plane, and the following devices were implanted (bregma serving as the anterior/posterior [A/P] and lateral [L] references, and skull surface as the vertical [V] reference [units expressed in millimeters]): 1) EEG screw electrodes into the skull at coordinates (a) A6.0, L2.0, (b) P8.0-10.0, L3.0, and (c) ground reference screw behind lambda. 2) Nasal bone screw electrodes at (a) A20.0, L2.0, and (b) A40.0, L3.0. 3) Lesion electrodes (Formvar-coated stainless steel, 250 μm outer diameter, insulated except for the tip) at A1.0-1.5, L1.5 mm, V13.5; one electrode was implanted on each side. The lesion electrodes were fixed in place with dental cement, which also anchored the EEG screw electrodes. 4) A thermistor (Fenwall Elect. No. GA45J1, 50K) insulated with dental cement was lowered at P8.0-10.0, L3.0 into cortical tissue. 5) Finally, a guide tube for lateral ventricular infusions was fixed as described before (77) at P3.0, L3.0. All leads from the EEG screw electrodes, implanted lesion electrodes, and thermistor were connected to a plug (Amphenol No. 223-1509), each lead being soldered to an individual pin (Amphenol No. 220-P02). The plug also was secured to the skull with dental cement.

After surgery, each animal was injected intramuscularly with Gentamicin sulfate (Tech America, KS) and returned to the animal room where food and water were available ad libitum. Ambient temperature (T_a) was kept at 20-23°C. Seven to 14 days were allowed for recovery before any measurements were started.

When animals received ICV injections, MDP (150 pmol) was dissolved in 25 μl PFS and infused slowly over 5 min into a lateral ventricle. When MDP was injected into a marginal ear vein, 25 μg/kg was prepared in 0.5 ml of PFS. These two doses elicit similar sleep and thermal responses (77). The bioassay described above was used.

2. Specific Protocol. Before lesions were made, data were obtained from all the animals without treatment (no infusion), and on subsequent days after the administration of MDP. The "no infusion" treatment was adopted to establish control values in order to minimize the number of injections each animal would receive. Previously, we showed that the administration of either PFS or the inactive stereoisomer of MDP, MDP-DD, did not alter subsequent sleep patterns relative to those observed in untreated rabbits (146). After establishing baseline values, the animals were subjected to the lesion procedure. After overnight adaptation in the recording chamber to an T_a of 20-21°C, T_a was lowered to 5°C. Two hours later, the conscious rabbit was placed in a restraining box, and the lesion was made bilaterally by passing direct current (3 mA, 30-35 sec; Grass lesion maker #DC LM5A) between the implanted lesion electrode (anode) and the tip of the ear pinna (cathode). The animal was then returned to the recording chamber for 10 h at 5°C. The chamber temperature was then elevated to 15°C for the rest of the "lesion day." On postlesion day 1, T_a was gradually brought up to 20-21°C. This procedure was modified from that of Nagel and Satinoff (111) and increased the survival rate to about 80%. Additional special care was also provided. Thus, all animals received daily fresh cabbage and carrots after the lesion to entice them to eat. In some cases, it was necessary to directly offer this food to their mouths before they would eat. Two days after placement of the

lesion, the animals were tested again without treatment, and on day 3 following MDP administration. If febrile responses to MDP were greater than 42°C, T_a was reduced after the recording period. Finally, between 8-14 days after the lesion, the animals were again tested without treatment, and between postlesion days 9-15 after MDP administration. Of the 22 animals used in this study, five did not survive long enough to be tested with MDP on postlesion day 3; data obtained from these rabbits were excluded. Another five animals were found post facto histologically to have sustained no damage (n = 4) or minor unilateral damage to the POA (n = 1). The data obtained from this group were treated separately. Of the remaining 12 animals, all showed bilateral damage to the POA (see Results), and all received MDP on postlesion day 3; eight of these animals also received a second MDP treatment between postlesion days 9-15. Data from these 12 animals constitute most of the results reported herein.

In most cases (10 of the 12 animals used in this study), a somnogenic dose of MDP (150 pmol) was administered ICV. In two cases, MDP was administered IV both pre- and postlesion. Since the data obtained after ICV injection did not substantially differ from those after IV injection, they were pooled for group analyses.

3. Histology. After completion of the experiments or when an animal's condition deteriorated during the experiment (e.g., did not accept cabbage or carrots, showed declining T_{co}, was lying down without support), animals were sacrificed using an overdose of pentobartibal administered into a marginal ear vein. Saline and, subsequently, 10% formalin were perfused through the heart. The brain was removed and placed in 10% formalin for 7-10 days, then sliced in 40-μm-thick sections and stained with cresyl violet (7).

C. Experiment No. 3: Effects of Staphylococcus aureus infections on rabbit sleep

Adult male New Zealand White rabbits (Pasteurella- and coccidia-free) weighing 4-5 kg were surgically implanted with EEG recording electrodes and brain thermistors as described above (83). In all experiments, 2 rabbits were tested simultaneously. Prior to the experiments, they were moved to experimental cages and were allowed an overnight period of adaptation. Baseline sleep patterns were recorded for 24 hr before the animals received any experimental treatment. The animals then were inoculated IV with Staphylococcus aureus (S. aureus) at 08:00 hr (time 0 on figures), as described below, and recording was continued for an additional 48 hr. Blood samples and T_{co}s were taken at 6- or 12-hr intervals throughout this period. In one experiment, both rabbits received S. aureus inoculations, but one also received the antibiotic cephalothin (40 mg/kg, i.m.), and the other an appropriate volume of saline vehicle. These latter injections were repeated every 12 hr.

EEG and T_{br}s were recorded via a rotary commutator (BRS-Tech Serv). Movement of animals was monitored using a Grass acceleration transducer connected to the EEG cable. The EEG signal was electronically filtered, and the delta wave component (0.5-4.0 Hz) was rectified using a Buxco EEG analyzer (Buxco Electronics, Sharon, CT) (77). These recordings were displayed on a Grass polygraph. The average EEG delta wave amplitude was also recorded in digital form for each 1-min interval.

Periods of SWS are associated with increased amplitude of low frequency (delta) EEG waves and with the absence of body movement. On this

basis, the EEG tracing, the filtered and rectified EEG delta wave signal, and the movement record were visually examined over the first 6 hr of the baseline recording period to determine the threshold amplitude of the delta waves associated with SWS for each animal. Each animal's data was then scored in 1-min intervals for the entire experiment; the animal was considered to be in a state of SWS whenever the average delta amplitude for any interval exceeded the SWS threshold amplitude in the absence of movement by the animal. At other times, the animal was either awake or in REMS. REMS was identified in some animals by visual assessment of the polygraph record, based on the criteria of low voltage EEG, a rise in T_{br}, and the sporadic occurrence of phasic body movement (83). Data were analyzed throughout the recording period and summarized for every 2-hr interval. Sleep parameters evaluated included the percentage of time spent in SWS, the average EEG slow wave amplitude during SWS, the average length of a bout of SWS, and the number of minutes spent in REMS during each 2-hr interval.

For the preparation of bacterial inocula, S. aureus (American Type Culture Collection 29213) was purchased as a lyophilized culture on Colti-loops. Prewarmed blood agar plates were inoculated and incubated overnight at 37°C, and colonies then transferred to sterile phosphate-buffered saline (pH 7.4) to achieve a concentration of approximately 2×10^9 colony-forming units (CFU) per ml, estimated using a Klett-Summerson photoelectric colorimeter. The number of CFU per ml of inoculum was later verified by plating serial dilutions of the bacterial suspension on blood agar plates and counting colonies after a 24-hr incubation at 37°C. In experiments using heat-killed bacteria, suspensions were autoclaved prior to animal inoculation and were later confirmed to be free of live bacteria by incubation on blood agar

plates. Rabbits were inoculated IV in the marginal ear vein with 0.1-2.0 ml of the bacterial suspension. Each rabbit was inoculated with S. aureus only once and was sacrificed at the end of the recording period.

Blood samples (1-2 ml) were collected from the central ear artery and were immediately transferred to Vacutainer tubes containing EDTA. Total white blood cell (WBC) counts were measured using a Coulter model 2N cell counter. Differential WBC counts were made by counting 100 white cells from blood smears stained with Wright's stain; final WBC counts were corrected for nucleated red blood cells (nRBCs), if present. Plasma cortisol levels were measured using a radioactive immunoassay kit (Kallestad Labs, Austin, TX).

Data were analyzed using two-way analysis of variance for repeated measures, with individual means compared using Fisher's Least Significant Difference Test for a priori comparisons. A significance level of $p < 0.03$ was used.

D. Experiment No. 4: Somnogenic activities of synthetic Lipid A

Test materials. Synthetic E. coli-type lipid-A (compound LA-15-PP), its monophosphoral analog (compound LA-15-PH) (Fig. 11), and a diphosphoral tetraacyl biosynthetic precursor Ia to these compounds (LA-18-PP) were tested. In addition, a synthetic Salmonella minnesota-type monophosphoral lipid A, compound LA-16-PH (Fig. 11), was used. These substances were synthesized and characterized for certain biological activities as previously described (57, 58). Lipid X, a diacyl monosaccharide component of lipid A, was a gift from Dr. N. von Jemey of Sandoz Research Institute. All samples were

dissolved or suspended as homogenously as possible at 1 mg/μl in 0.1% triethylamine (vol: vol) aqueous solution. Compounds 15-PP, 13-PP, and lipid X completely dissolved, whereas compounds 16-PH and 15-PH were less soluble and produced a slightly turbid suspension. Appropriate aliquots were then further diluted in PFS before injection into animals; after these dilutions, all solutions were transparent. Rabbits did not receive two IV injections of the same material, nor were they used again for at least 1 wk after any assay. No signs of tolerance to lipid A were observed under these conditions. Control recordings were taken on different days using identical procedures, except that no materials were administered, providing a matched-pairs experimental design. Previously it was shown that ICV or IV injections of control substances do not alter subsequent sleep in rabbits (87).

The percentage of time spent in each vigilance state (SWS, REMS, W) was calculated for each hour for each rabbit, and average EEG voltages were calculated similarly. Treatment groups were compared to their own controls with a Friedman test (nonparametric 2-way ANOVA) to determine possible treatment effects. If significant differences were found, the Wilcoxon matched pairs test was used to determine when the differences occurred. In some cases, additional analyses were performed using data collected only during postinjection hours 2-4 (for IV injections) or 3-5 (for ICV infusions). This was done because previous work with lipid-A molecules showed that somnogenic effects were maximal during these periods (83).

E. Experiment No. 5: Effects of αMSH on sleep, behavior and brain temperature: interactions with IL1

αMSH was purchased from Peninsula Laboratories, Inc. (Belmont, CA). IL1 (human recombinant IL1β) was kindly provided by Dr. C. Dinarello of Tufts University, School of Medicine.

1. Experimental Protocol

αMSH: Approximately 3 h after light onset, rabbits were injected ICV with either aCSF for control recordings or with one of four doses of αMSH (0.1 μg, n = 8; 0.5 μg, n = 8; 5.0 μg, n = 8; or 50.0 μg, n = 7). The injection (25 μl) was administered over a 2-min period. Control and test recordings were separated by one week.

IL1 and IL1 + αMSH: Substances used in experiments to determine the effects of IL1 and IL1 + αMSH were admnistered ICV as double injections following procedures reported by Lipton et al. (21, 93). Combinations were tested as follows: 1) Two injections of aCSF (12.5 μl) separated by 30 min constituted control recordings. 2) When the effects of IL1 were tested, IL1 (20-40 ng) was ICV injected, followed 30 min later by aCSF. 3) Finally, in experiments with IL1 + αMSH, αMSH was injected 30 min after IL1 in these combinations: 40 ng IL1 + 0.1 or 0.5 μg αMSH, and 20 ng IL1 + 0.5 or 5.0 ng αMSH. Eight rabbits were used for each combination. Doses of αMSH and IL1, the latter as estimated from pyrogenic action, correspond to those reported by Lipton et al. (21, 93).

2. Behavioral Observations. Effects of αMSH on rabbit behavior were determined by using a closed-circuit TV system. Rabbits were placed in recording chambers identical to those used for electrophysiological recording except the door was replaced by a Plexiglas panel. Animals were allowed to become accustomed to the recording chamber for a minimum of 30 min, after which they received ICV injections of aCSF, αMSH (0.5 or 5.0 μg), or IL1 (20 ng) + αMSH (0.5 or 5.0 μg) as described for the sleep studies. Their behavior was videotaped for 1 h. The tapes were visually scored at 1-min intervals and the rabbits' behavior classified as inactive (sitting or lying without body movement), active (any movement about the chamber, e.g., exploration, rearing), grooming, or ingesting (eating or drinking). In addition, each occurrence of stretching/yawning (stretching or yawning separately or the combination of both) and sexual excitation (operationally defined as copulatory movements consisting of rapid pelvic thrusting) was scored as a discrete event.

3. Statistical Analysis. Data from all experiments were analyzed with SPSS^X Information Analysis System. The Wilcoxon matched-pairs signed-ranks test and Friedman's test for k-related samples were used to determine statistical significance as indicated. The medians test was used to determine if the median duration of W episodes changed between control and experimental periods. Behavioral data were analyzed with the Wilcoxon matched-pairs signed-ranks test (gross behavior) or Chi-square tests (discrete events).

F. Experiment No. 6: Effects of GRF on rat and rabbit sleep

1. Experimental procedures in rats. Male CFY rats (300-350 g) were used. Surgery and experimental procedures followed the methods described

previously (68, 109). Under pentobarbital anesthesia (50 mg/kg), gold jewelry screws were implanted over the frontal and parietal cortices and the cerebellum for EEG recording. Diodes cemented over the parietal cortex served for T_{br} measurements. An ICV guide cannula was implanted into the left lateral ventricle. Stainless steel 23-gauge needles were used for the ICV injections. The placement of the cannula and the drainage of the ventricle were tested in vivo by means of the drinking response elicited by angiotensin. Angiotensin (100 ng, 1 µl) injected ICV elicits drinking in about 2 min via the stimulation of preoptic structures (37). Each animal was tested five days before and immediately after the sleep-wake recordings. After termination of the experiments, trypan blue was injected ICV, and cannula placement was checked in frozen sections of the brain. Data were used only from those rats in which the angiotensin test was positive both before and after the experiments, and the examination of brain sections confirmed the proper position of the cannulas.

Animals were adapted to the experimental conditions for 7-10 days after the surgery. During this period, they were connected to flexible recording cables in individual Plexiglas cages in sound-attenuated recording chambers. Continuous low-level noise was provided. The rats were housed on a light-dark cycle of 12 h each, with lights on from 08:30 to 20:30 at a T_a of 21 ± 1°C. The same light-dark cycle and T_a were provided in the experimental chambers.

The animals received an ICV injection of artificial cerebrospinal fluid (aCSF) (78) once each day for five days before the experiment. When GRF was injected, it was dissolved in aCSF. GRF or aCSF solutions were

injected 5-10 min before dark onset in a volume of 2-3 μl over a 1-2 min period. Half of the animals in each group were injected with aCSF on day 6, and the EEG, motor activity, and T_{br} were recorded for 24 h. The next evening, GRF was administered, and the recording was continued for another 24 h. The injection sequence of aCSF and GRF was reversed for the other animals, i.e., the GRF day preceded the baseline (aCSF) day.

2. Evaluation of data in rats. An automatic analyzer using a modified program developed by Neuhaus and Borbely (113) scored the vigilance states as W, SWS, and REMS for each 10-sec period. The program evaluated the EEG signal and motor activity (potentials generated in electromagnetic transducers activated by the movement of the recording cable) as described previously (68). W was characterized by a relatively high level of theta [6-10 Hz] activity, low EEG amplitudes, and motor activity. SWS was identified by high EEG amplitudes, low level of theta activity, and lack of motor activity. REMS was associated with relatively low EEG amplitudes, theta activity exceeding that in W, and lack of motor activity with occasional twitches. Motor activity counts were integrated for 10-sec periods, and T_{br} values were taken at 10-sec intervals. The scores of sleep-wake states, integrated motor activity counts, and T_{br} were stored on a magnetic tape and later processed to obtain the percentages of the sleep states, motor activity counts, and T_{br} values for consecutive 1-h and 3-h periods. The differences in motor activity counts, T_{br}, and percentages of the vigilance states were compared between the GRF day and the baseline day. The paired Student's t-test (two-tailed) was used for statistical evaluations of the results.

3. Experimental procedures in rabbits. The methods and experimental schedules used were as described above. The percentages of the vigilance states were calculated for 1-h and 6-h periods. The differences between the GRF day and the baseline day were evaluated by using the Wilcoxon matched pairs signed ranks test.

4. EEG analysis. Spectral analyses were used to evaluate the effects of GRF on EEG activity in the first 3 h of the recording, i.e., at the beginning of the dark period, as described previously (68). Briefly, after analog-digital conversion, the EEG signals (0.53 Hz - 30 Hz at 6 db/octave) were processed through fast fourier transformation. Power density values ($\mu V^2/0.4$ Hz) were computed for 2.4-sec periods. To characterize EEG slow-wave activity, the spectra were averaged for 2-Hz frequency ranges (0.4-2.0 Hz, 2.4-4.0 Hz, and 4.4-6.0 Hz). The mean power densities were calculated for consecutive 10- and 30-min periods within these ranges. The differences between the power density values on the GRF night and baseline night, expressed as percentages of the baseline power densities, were evaluated using paired Student's t-tests (two-tailed).

In rabbits, the average amplitudes of the EEG activity calculated for the frequency bands described above were determined. The average amplitude values were calculated for each postinjection hour and for each frequency range. Further, the average amplitudes were also computed for 1-min periods during W, SWS, and REMS in postinjection hour 1. For each rabbit, the voltages from 10 periods of uninterrupted SWS with the highest voltages were averaged. For REMS and W, the voltages from all uninterrupted periods from each rabbit were used because there were relatively few periods of 1-min

duration. The differences between the amplitude values obtained after GRF injections and those obtained on the baseline day were evaluated using the Wilcoxon matched pairs signed ranks test (two-tailed).

5. Peptides. Human GRF(1-40) was used in these studies. The GRF injected into rats was provided by J. Rivier (Salk Institute, San Diego, CA); the GRF samples administered to rabbits were purchased from the Peninsula Laboratories, Inc. The doses of GRF tested on the various groups of animals were 1 nmol/kg (rats: n = 8; rabbits: n = 6), 0.1 nmol/kg (rats: n = 10; rabbits, n = 8), and 0.01 nmol/kg (rats: n = 13; rabbits: n = 7).

RESULTS

Experiment 1

Structure determination: The chemical structures of the tested substances as were determined by FABMS are depicted in Table 1. It should be noted that FABMS does not provide information about steric confirmations of chiral centers. However, since the MPs are derived from bacteria, it can be assumed that the peptide sequence is in the form L-Ala-D-Glx-LL or Meso-DAP (with the Cα in the peptide chain in L-form)-D-Ala-D-Ala (100).

A. Effects of monomeric MPs

1. Effect of O-acetylation of muramic acid. The effects on SWS, REMS, and temperature of three pairs of MP structures were compared (#1 to #6). The substances were different only in that one MP was substituted with

an O-acetyl group in the C6 position of its muramic acid (#1, #3, #5), while the other MP of the respective pair was not (#2, #4, #6). The effects of the non-O-acetylated MPs have been published previously (87) and their effects on sleep and temperature are included here for ease of comparison.

The O-acetylation of muramic acid in NAG-(6,OAc)NAM-Ala-γGlu-Dap-Ala (#3) reduced the dose needed for a significant effects on SWS as compared to the respective non-O-acetylated compound (#4). The administration of 10 pmol was sufficient to enhance SWS. This makes the effect comparable to the effect found with 1-6-anhydro substitution of NAM in MPs (87), although a 10-fold higher doses was needed.

2. Substance #7 was substituted both with an effect-enhancing (1,6-anhydro-NAM) and an effect-preventing group (the free amide on the terminal alanine). As could be expected from previous data, the unsubstituted amidation of the terminal carboxyl group in NAG-(1,6 anhydro)NAM-Ala-γGlu-Dap-Ala (NH2) (#7) prevented the SWS-enhancing activity of this molecule (87). However, a significant effect on T_{co} temperature was noticed when 100 pmol of this substance was infused.

3. If the MP was only amidated as in MP #8, no influence on sleep and temperature parameters were found, confirming previous observations with other MPs on the biological effect preventing influence of this structural feature (80).

B. Effects of dimeric MPs

1. Dimers connected via peptide cross-linkage: Three different MP dimers were available (#9, #10, #11) which were built from monomers connected by an interpeptide bridge of the type known to occur in Neisseria gonorrhoeae. One dimer consisted of two NAG-NAM-Ala-γGlu-Dap-Ala molecules connected via a peptide linkage from the γ-amino group of Dap of one monomer to the COOH-group of the terminal Ala of the other monomer (#9). This MP had no SWS-promoting activity at the concentrations tested, although the respective monomer (#4) was found to be somnogenic at 100 pmol. The addition of a terminal alanine to the carboxyl end of the monomer containing the free ala of compound #9 to give compound #10 did not change this finding. It is not known so far if a monomer NAG-NAM-Ala-γGlu-Dap-Ala-Ala is somnogenic. However, the introduction of 1,6-anhydro substitution of NAM in MP dimer #9 as in dimer #11 rendered this substance somnogenic and pyrogenic at 100 pmol. A pyrogenic effect could be observed at a lower dose of 10 pmol.

2. Dimers connected by β-1,4-glycosidic bond: Four MP dimers were available to us which consisted of monomers connected in their sugar moieties via glycosidic linkage. Two of the glycosidically linked dimers (#12, #13) contained free terminal amino groups and were inactive even though one contained a 1,6-anydro muramic acid (#12). Two other MP-dimers (#14, #15) were found to be somnogenic and pyrogenic after injection of 100 pmol. On both these substances it was not determined whether the amidation on the terminal Glu occurred on the α- or the γ-C atom. The substitution of one of the constituting monomers as in MP-dimer #14 made this substance SWS-promoting and REMS-suppressing even at 10 pmol.

Experiment 2

Extent of the Lesions. The extent of tissue damage was different for each animal, although all animals (n = 12) that were used for data analyses showed bilateral damage to the POA. In general, lesions were located in an area extending anteriorly from the diagonal band of Broca to the anterior hypothalamus (AH). Only two animals had lesions extending into the AH; in both cases, damage was unilateral and extended only about 0.5 mm into the AH from the POA. Dorsally, lesions extended from the anterior commissure to the optic chiasm (OC). Four animals had small (maximum, 0.2 mm deep) unilateral damage to the OC, while in two animals, the OC was extensively damaged. Of the 12 animals, most showed some damage to the medial and lateral preoptic regions, the diagonal band of Broca, and the medial forebrain bundle (e.g., Fig. 2). Of the five animals that did not survive long enough to obtain EEG recordings three days after the lesion, histological sections were not obtained from two of these animals. The other three had large lesions in the POA extending through the third ventricle. Another four animals had no damage to the POA. In three of these animals, no damage was evident in the sections we prepared; in the other animals, damage was dorsal to the anterior commissure and extended into the cortex. In another animal, there was a small unilateral lesion (about 0.5 mm in diameter) in the lateral POA.

Effects of the Lesions

Sleep: EEG recordings were obtained from five animals which either did not have damage to the POA (n = 4) or had minor unilateral damage in the POA (n = 1). These animals did not exhibit alterations of SWS; thus, pre-

lesion duration of SWS was 45 ± 2% vs. postlesion values of 44 ± 2%. As a group, duration of REMS also was not significantly altered: 8.1 ± 2.2% and 4.1 ± 1.4%, before and after the lesion, respectively. However, three of these five animals had only one-fourth or less REMS than that observed before the lesion.

The remaining 12 animals had bilateral damage to the POA and thus were used for further data analyses. The different structures that were ablated in these rabbits, the limited number of animals, and the normal variation in the amount of time rabbits spend in sleep all precluded correlating their specific damage to the extent of insomnia observed after the lesion. However, two rabbits that slept the least on postlesion day 2 (7 and 10% SWS) showed the most extensive bilateral damage to the POA, while, by contrast, three animals that slept the most after lesion (33, 33 and 44% SWS) had relatively slight damage to the POA. Duration of SWS and damage to the POA in the remaining animals varied between those ranges.

On the second day after POA lesions were made, all 12 animals showed reduced duration of SWS compared to prelesion values: average values were 53 ± 2% prelesion as compared with 24 ± 3% post-lesion (Table 2). This reduction was due primarily to a reduced duration of SWS episodes. The average number of SWS episodes was also fewer two days after than before the lesion, but this difference was not statistically significant (Table 2). Measurements were taken from eight of these animals 8-14 days after the lesion, without treatment; at this time, the duration of SWS had recovered to the extent that it was not significantly different from prelesion values (Table 2). However, the duration of SWS episodes remained about half of that observed before the

lesion, and the number of SWS episodes observed at this time was slightly, but not significantly, greater than their prelesion values.

Two days after POA lesion, EEG slow-wave voltages during SWS were about 56% of prelesion values (Table 2). In contrast to the recovery of the duration of SWS, these reduced amplitudes of EEG slow waves during SWS persisted 8-14 days postlesion.

The effects of POA lesion on REMS were similar to those on SWS. REMS was significantly reduced two days after the lesion and remained significantly lower than prelesion values 8-14 days after the lesion (Table 2). Six of the 12 animals that had POA lesions did not have any REMS during the recording sessions. Since one effect of MDP is to inhibit REMS (see below), REMS episodes were not analyzed in those animals.

After the lesions, EEG, EEG-theta/delta ratios, movement, and T_{br} continued to fluctuate in conjunction with states of vigilance in a manner similar to that observed in prelesion recordings. Thus, the criteria used for state identification remained valid after the lesion (Fig. 1).

Behavior: POA-damaged rabbits displayed fear reactions more often than normal rabbits, e.g., foot stomping, sudden forward running, and shrieking. These were elicited by sudden environmental events and by handling, such as required during measurement of T_{co}. There was, however, no striking correlation between these tendencies and sleep disturbances; i.e., at least some rabbits with such hyperreactivity displayed relatively normal sleep patterns.

Temperatures: Lesion of the POA also had a profound effect on T_{co} (Table 2). Two days after the lesion, T_{co}s were elevated in all animals, ca. 1.4° C above prelesion values (Table 2). T_{co}s remained significantly elevated on postlesion days 8-14, although the magnitude of hyperthermia at this time was less (0.7°C) than that observed two days after the lesion. By contrast, the T_{co}s of the animals that did not have damage to the POA were not altered by the procedure (prelesion, 39.0 ± 0.1°C; postlesion, 39.4 ± 0.2°C).

Effects of MDP

Sleep: Prior to the lesions, MDP induced increases in duration of SWS, decreases in duration of REMS, and elevations in T_{co} (Table 2; Figs 3 and 4), thus confirming previous findings (77, 146). The enhanced SWS resulted from an increase in the number of SWS episodes (Table 2); the duration of individual SWS episodes was not altered. The amplitude of EEG slow waves during SWS was also enhanced after MDP injection into prelesion animals, thus also confirming previous results (77). Although behaviors other than sleep were not systematically measured, no gross behavioral abnormalities were observed in response to MDP either before or after lesion.

After POA lesion, the direction and magnitude of these MDP-induced responses were similar to those observed prior to the lesion, although the baseline values upon which these changes were superimposed had shifted (Figs. 3 and 4). Thus, after lesion, during the 6-h recording period, animals spent about 1 h more in SWS after MDP treatment than during postlesion control periods. On postlesion day 3, MDP-enhanced SWS resulted from an increased

number of SWS episodes; the duration of SWS episodes which was reduced by the lesion remained so after MDP treatment. On postlesion days 9-15, the duration of SWS episodes after MDP treatment significantly rose above control values taken the day before, although they were still not as great as pre-lesion values (Table 2). The number of SWS episodes after MDP treatment measured 9-15 days postlesion was also increased, but these values were not significantly above control values obtained the day before.

Amplitudes of EEG slow waves during SWS were also enhanced by MDP treatment three days and 9-15 days after the lesion (Table 2), although the increases were significant only on days 9-15 postlesion. Similarly, duration of REMS was less after MDP treatment than during the control recording taken the day before; the MDP-induced suppression of REMS was only significant 9-15 days postlesion. However, as mentioned above, on postlesion day 2, six of the 12 animals did not have any REMS, and on day 3, MDP did not induce any REMS in these animals. In those animals (n = 6) that had REMS, it was less on day 3 (after MDP injection) than on day 2 (Fig. 3).

The time courses of MDP-induced sleep effects were also similar before and after lesion (Fig. 4). During the first hour after MDP injection, SWS and REMS were not significantly different from corresponding control values. MDP-enhanced SWS became evident during the second postinjection hour, then persisted throughout the remaining 4 h of the recording period. MDP-induced REMS suppression was evident in all experimental groups during the last 4 h of recording. However, on postlesion day 3, the suppression of REMS during this 4-h period was not significant due to the large number of animals (n = 6) that had no REMS during the control recording on day 2.

Temperatures: MDP also induced febrile responses in the animals with POA lesions despite the fact that their T_{co}s were already elevated (Table 2). T_{br} changes normally associated with changes in states of vigilance also persisted in MDP-treated lesioned animals. Thus, after MDP treatment on postlesion day 3, decreases in T_{br} upon entry into SWS were observed, while rapid increases in T_{br} occurred in those few animals that had some REMS after their entry into the state. The magnitudes of these state-coupled T_{br} changes were small (0.05-0.1°C) before and after lesion as compared to T_{co} changes associated with MDP treatment or lesion (1.0-1.5°C).

Experiment 3

Effects of S. aureus infection on sleep, body temperature, and hematological parameters. Following IV inoculation of rabbits (n = 16) with 10^7 to 10^8 CFU (mean dose = $6.1 \pm 1.1 \times 10^7$ CFU) of viable S. aureus, the time spent in SWS increased during the period 6-18 hr after inoculation (Fig. 5a). The enhanced SWS was associated with increases in EEG slow wave amplitude during SWS (4-8 hr after inoculation; Fig. 5b) and in the length of individual bouts of SWS (6-16 hr after inoculation; Fig. 5c). These alterations in sleep were followed by a period during which the amount of SWS (26-34 hr after inoculation, Fig. 5a), EEG slow-wave amplitudes (20-36 hr after inoculation; Fig. 5b), and SWS bout length (24-36 hr after inoculation; Fig. 5c) were all significantly reduced relative to baseline values obtained at the same time of day.

Twelve rabbits that were inoculated with S. aureus had been implanted at the time of surgery with brain thermistors to allow the measurement of REMS.

During the baseline recording period, rabbits exhibited a circadian pattern in the occurrence of REMS, with relatively more time spent in REMS during the period in which the lights were on than during the lights-off period (Fig. 6). S. aureus inoculation markedly inhibited REMS from 6-42 hr after inoculation (Fig. 6).

SWS was also monitored in rabbits that were inoculated with 8×10^7 (n = 8) or 7×10^9 (n = 12) CFU of heat-killed S. aureus. The lower dose, which contained approximately the same number of organisms as the viable inoculum, did not significantly alter any of the sleep parameters examined (Figs. 5d-f). In contrast, inoculation of rabbits with the higher dose of killed organisms significantly increased the time spent in SWS (2-6 hr after inoculation; Fig. 5d), EEG slow wave amplitudes (2-4 hr after inoculation; Fig. 5e), and SWS bout length (2-8 hr after inoculation; Fig. 5f); following these effects, the EEG slow wave amplitude was significantly decreased from 22-30 hr after inoculation (Fig. 5e). Thus, the effects of inoculation with the higher number of killed S. aureus organisms were qualitatively similar to those produced by inoculation with viable organisms, although the sleep alterations induced by the high doses of killed organisms were characterized by a more rapid onset and shorter duration than were the effects of the viable inoculum.

Inoculation of rabbits with viable S. aureus was accompanied by a 1-1.5°C increase in T_{co} from 6-48 hr after inoculation (Fig. 7). The febrile effects of bacterial inoculation could thus be dissociated temporally from the somnogenic effects. Inoculation with the same number of heat-killed organisms did not significantly alter T_{co}, although at the higher dose, rabbits were febrile at 6 and 12 hr after inoculation (Fig. 7).

Neutrophilia, lymphopenia, and elevated plasma cortisol levels are commonly associated with inflammatory, infectious, or stressful conditions in animals, and circulating nRBCs can be associated clinically with septicemia (41, 61); thus, these hematological parameters were examined after either viable or heat-killed S. aureus inoculation. Both viable organisms and the higher dose of killed organisms produced marked neutrophilia from 6-48 hr after inoculation; the lower dose of killed organisms produced significant neutrophilia only at 12 hr after inoculation (Fig. 8a). All three inocula resulted in marked lymphopenia 6 hr after inoculation; this effect persisted until 12, 36, and 48 hr after inoculation with the low dose of killed organisms, the high dose of killed organisms, and the viable organisms, respectively (Fig. 8b). The number of nRBCs present in peripheral blood also increased from 12-48 hr after inoculation with viable organisms; this effect was not observed following inoculation with killed organisms (Fig. 8c). Inoculation either with viable organisms or with the high dose of killed organisms resulted in significant increases in plasma cortisol levels from 6-24 hr after inoculation (Table 3). Postmortem blood cultures from animals that received viable S. aureus revealed circulating gram-positive bacteria in 10 of 14 animals tested.

Effects of cephalothin administration on S. aureus-induced alterations in sleep, fever, and hematological parameters. The bacteriocidal antibiotic cephalothin, which is known to inhibit growth of the strain of S. aureus used in these experiments (46), was administered to rabbits to evaluate the effects of antibiotic therapy on sleep patterns during infection. One group of rabbits ($n = 8$) was inoculated with viable S. aureus (5×10^7 CFU) and vehicle (saline). Significant time-dependent changes in the time spent in SWS, EEG slow wave amplitudes, and SWS bout length were observed in this group (Fig.

9a-c), as were observed with the group described above (Fig. 5). Another group of animals (n = 8) received cephalothin (40 ng/kg, i.m., q 12 hr) in conjunction with S. aureus inoculation. Cephalothin markedly attenuated the effects of S. aureus inoculation on the time spent in SWS, although significant increases in SWS still occurred from 6-16 hr after inoculation (Fig. 9a). Cephalothin did not attenuate the initial stimulatory effects of S. aureus inoculation on EEG slow wave amplitudes, but it did eliminate the subsequent inhibitory effects (Fig. 9b). Both the initial increase and the subsequent decrease in SWS bout length were attenuated by cephalothin (Fig. 9c). Cephalothin also shortened the duration of S. aureus effects on body temperature, neutrophil and lymphocyte numbers, and plasma cortisol levels (Fig. 10a-d). A third group of animals (n = 6) received cephalothin alone; this treatment did not significantly alter any of the parameters examined (Figs. 9, 10).

Relationship of sleep patterns to severity of infection. Six of the 24 rabbits that received S. aureus inoculations in the preceding experiments died within 24 hr after inoculation (n = 3) or were sacrificed 12 hr after inoculation because of a moribund condition characterized by extreme behavioral depression with unresponsiveness to handling and other stimuli, peripheral vasoconstriction as evidenced by cold extremities, and dyspnea (n = 3); these six rabbits will subsequently be referred to as Group I. The remaining 18 rabbits (Group II) were affected less severely and remained clinically stable for up to 48 hr after inoculation. The severity of the clinical response was related to the dose of S. aureus administered (mean doses administered to Group I and Group II were $8.6 \pm 2.5 \times 10^7$ and $4.9 \pm 0.6 \times 10^7$ CFU respectively; $p < 0.01$). The sleep patterns of animals that succumbed to the infection

(Group I) were compared to patterns of surviving animals (Group II) during the initial 12 hr after inoculation. Animals in Group I slept substantially less than those in Group II from 10-12 hr after inoculation and also demonstrated decreased EEG slow wave amplitude and SWS bout length from 8-10 hr after inoculation (Table 4). Group I rabbits also exhibited a more rapid rise in T_{co}, nRBCs, and plasma cortisol levels than did Group II rabbits (Table 5). Changes in lymphocyte numbers were similar between the two groups; however, Group I animals failed to exhibit a strong neutrophil response (Table 5).

Experiment 4

Compound LA-15-PP. Compound 15-PP, a hexaacyl diphosphoral lipid A (Fig. 11), was the most active compound tested in this study. After either 0.03 μg/kg or 0.3 μg/kg administered IV, significant increases in SWS for the 6-h recording period were observed (Table 6). The greatest increases in SWS were observed 2-4 h after IV injection; whereas after ICV administration, the largest effects on SWS were observed 3-5 h after injections, confirming previous results with other lipid-A substances (83). Thus, if we restrict the analysis to hours 3-5 after ICV administration, both doses tested ICV (1 and 10 ng) significantly enhanced SWS; values were (± standard error of mean) 34 ± 4 control and 45 ± 4 experimental after 1 ng, and 47 ± 3 control and 53 ± 4 experimental after 10 ng.

Quantification of average delta-wave EEG voltages (slow wave; 0.5-3.5 Hz) is a sensitive electronic method that may be used as an objective measure to amplify and verify visual scoring of EEG records (145). Significant increases of EEG delta amplitudes were found for all the doses tested after compound

15-PP was given either IV or ICV (Table 6). The time course of effects on EEG slow-wave voltage was similar to that observed for SWS, although both IV and ICV injections of compound 15-PP enhanced amplitudes of EEG slow waves during the first hour postinjection (Fig. 12).

IV administration of compound 15-PP induced decreases in REMS. After the lower IV dose (0.03 μg/kg), three of the four animals tested had less REMS than that observed under control conditions, although this effect was not significant (Table 6). After the higher IV dose (0.3 μg/kg), a significant decrease in REMS was observed. In contrast, REMS was not significantly reduced for the 6-h recording period after either ICV dose. However, if we restrict the analysis to postinjection hours 3-5, the period during which maximum effects on SWS were observed, a significant decrease in REMS was evident after 10 ng ICV injection of compound 15-PP (6.2 ± 0.4 control vs. 2.9 ± 0.7 experimental).

Although rabbit behavior was not systematically quantified in these studies, compound 15-PP, at the doses tested, did not induce gross abnormal behavior. Thus, the animals continued to cycle through the three major states of vigilance in a relatively normal fashion, although durations of individual states were altered. Animals could easily be aroused and showed normal responses when T_{co}s temperatures were measured at the end of the 6-h recording period.

Colonic temperatures taken at the end of the 6-h recording period were also elevated after administration of compound 15-PP, although after IV injection only the higher dose induced significant increases (Table 6). The

courses of T_{br} responses after IV injections were different from those observed after ICV injections. Thus, after IV injections, T_{br} increased within the first hour, and by 3.5 h postinjection, values returned to near control values (Fig. 12). In contrast, ICV injection of compound 15-PP produced a relatively slow onset of elevated T_{br}, and maximum increases were observed at the end of 6 h. Another aspect of temperature regulation remained undisturbed after injection of compound 15-PP, that is, T_{br} changes that are coupled to states of vigilance (168) continued to fluctuate in a normal fashion (data not shown). However, it is noted that these vigilance-state-coupled changes in T_{br} are small in magnitude (0.1-0.3°) compared to the febrile responses induced by compound 15-PP.

Compound LA-15-PH. Compound 15-PH is the monophospholipid analog of compound 15-PP (Fig. 11) and, in general, had effects on sleep and T_{co}s that were similar to, although weaker than, those observed with compound 15-PP. Only the higher IV dose tested (3.3 μg/kg) significantly enhanced the duration of SWS and amplitudes of EEG slow-wave voltages (Table 6). Neither ICV dose of compound 15-PH enhanced duration of SWS. Both IV doses of compound 15-PH induced a significant decrease in REMS. REMS was reduced after both ICV doses, although neither effect was significant (Table 6). No abnormal behavior was observed after any dose of compound 15-PH.

The highest IV and ICV doses of compound 15-PH also induced increases in T_{co} at the end of the 6-h recording period (Table 6). The time course of T_{br} changes induced after IV injection (3.3 μg/kg) of compound 15-PH was biphasic (Fig. 13). Changes in T_{br} after IV injection of the lower dose (0.3 μg/kg) were minimal. It is important to note that, as mentioned above,

this dose significantly decreased REMS. T_{br} changes coupled to states of vigilance were not altered after administration of 15-PH.

Compound LA-18-PP. Compound 18-PP is a tetraacyl diphosphorlipid biosynthetic precursor of lipid A molecules (Fig. 11). This substance, when given IV (3.3 μg/kg), significantly enhanced SWS and EEG slow-wave voltages for the 6-h recording period (Table 6). Neither the lower IV dose (0.3 μg/kg) nor the two ICV doses significantly altered the duration of SWS. However, the higher ICV dose (10 ng) of compound 18-PP significantly enhanced EEG slow-wave voltage, although this effect was small (Table 6). The higher IV (Fig. 13) and ICV doses (Table 6) also enhanced T_{br} and T_{co}, respectively. As with the other compounds tested in this study, this substance did not induce abnormal behavior at the doses tested.

Compound LA-16-PH. Compound 16-PH, a septaacyl monophospholipid lipid A (Fig. 11), was almost devoid of any of the biological activities assayed in this study (Table 5, Fig. 14), even when tested using doses 10 times greater than those of the other three lipid A compounds. In addition, if we confined our analyses to hours 2-4 post-IV injection or to hours 3-5 after ICV injection (the periods during which maximum responses were observed with the other lipid A's tested), no significant effects were observed (data not shown).

Lipid X. Lipid X is essentially the diacyl monomer of a tetraacyl diphospholipid molecule (Fig. 11). It caused no significant changes in SWS when given IV, but it did significantly increase SWS after ICV administration of 100 ng (Table 6). The delta EEG voltages did not change across all IV or

ICV doses of lipid X (Table 6). Thus, the slight increase of SWS observed after the highest ICV dose was not accompanied by a concurrent increase of delta EEG voltages. No significant changes in REMs were observed after IV administration of lipid X, but a significant, although small, increase of REMS was induced by the 100 ng ICV dose (Table 6).

No significant changes of T_{co} ($\geq$ 0.5°C) were observed after administration of lipid X at any dose tested, IV or ICV (Table 6), nor did lipid X alter T_{br} changes that are coupled to states of vigilance.

Experiment 5

Effects of αMSH. ICV injection of αMSH elicited a decrease in T_{br} (Fig. 15). The time course of the temperature curves after αMSH injection differed from that after aCSF injection for each dose, including the small dose of 0.1 μg (Friedman's test across 6 h; $P < 0.01$). T_{br} started to decrease compared to aCSF by 10 min postinjection. Thereafter, the courses of the temperature curves varied with dosage; T_{br} after 0.1 μg αMSH remained slightly below control values throughout the recording period, whereas, after larger doses of αMSH, T_{br} initially dropped, then returned to control values in 3-5 h. The effect was dose-dependent (Friedman's test across 6 h; $P < 0.001$).

αMSH increased W (Fig. 16). The effects on sleep-wake activity depended on the dose (Friedman's test across 6 h; $P < 0.02$ for W, $P < 0.01$ for REMS, nonsignificant for SWS) and were most pronounced in postinjection hours 1 and 2. Apart from an increase in REMS in postinjection hour 5, the 0.1 μg dose

was ineffective in altering sleep-wake activity. After 0.5 μg αMSH, SWS was reduced and W increased in postinjection hour 1; REMS did not change. When 5.0 μg αMSH was injected, W increased for 2 h at the expense of both SWS and REMS. The largest dose of αMSH (50.0 μg) eliminated REMS for 5 h while reducing SWS and increasing W for 2 h. Sleep-wake activity was significantly altered across 6 h following 5 and 50 μg αMSH (Friedman's test; $\underline{P} < 0.05$).

The frequency of W episodes was studied as a function of their duration in postinjection hour 1. In response to αMSH, the median duration of W episodes increased due to an increase in the number of W episodes of long duration (longer than 4 min), while there was a tendency for the total number of W periods to decrease (Table 7). Continuous W for 1 h was observed in some rabbits after 50 μg αMSH.

EEG slow wave activity was suppressed in response to ICV αMSH during SWS episodes (if SWS occurred at all) in postinjection hour 1. A comparison of the average of the five greatest amplitudes in the delta frequency band for SWS episodes revealed significant decreases in slow wave amplitudes after 0.5, 5.0, and 50.0 μg αMSH, whereas the 0.1 μg dose was ineffective (Table 7).

Stretching/yawning was observed most frequently 11-20 min after administration of αMSH (Fig. 17 A, C: cumulative number of episodes). Stretching/yawning occurred with a mean frequency of one episode per rabbit during the 1-h observation period after ICV injection of aCSF. The number of stretching/yawning events increased to a mean of three and nine per rabbit in response to 0.5 and 5.0 μg αMSH, respectively. These increases were significant for both doses (Chi-square test; $\underline{P} < 0.02$ for 0.5 μg, and $\underline{P} < 0.001$ for 5.0 μg).

Sexual excitation was not observed after aCSF injection, and only one animal exhibited this activity after 0.5 μg αMSH (three occurrences). The cumulative number of episodes of sexual excitement increased after 5.0 μg αMSH (Fig. 17E) and appeared with an average frequency of two per rabbit; this effect was significant (Chi-square test; $P < 0.001$). The duration of stretching/yawning and copulatory movements was short (usually less than 1 min) and had little effect on other rabbit activities. No other unusual behaviors were observed.

The rabbits spent most of the time (close to 40 min) lying down during the 60 min after ICV aCSF injection. Grooming occurred occasionally for short periods, and ingestion occupied only a small fraction of time. The most frequently observed activity was exploration of the surroundings (moving around, rearing, sniffing). These activities sometimes lasted for several minutes, but were generally of shorter duration. The number of minutes when grooming or activity (which included stretching/yawning and sexual excitation) was observed did not change (Table 7); in fact, a tendency for increased inactivity was noted. Periods of ingestion were absent following 5 μg αMSH (Table 7). Qualitatively, the posture assumed when lying down seemed to be more extended, with slight changes in head, limb, or body position more frequent after both doses of αMSH than those observed during the control recording.

Effects of IL1

ICV administration of IL1 induced fever (Fig. 18). T_{br} started to rise shortly after IL1 injection with increases of 0.4°C and 0.5°C 30 min after the administration of the two doses (time 0 in Fig. 18). T_{br} rose steadily for about 2 h, with maximum elevation of 1.5°C and 2.4°C after 20 ng

and 40 ng IL1, respectively, and remained elevated for the entire recording period compared to T_{br} after aCSF (Friedman's test across 6 h; $P < 0.01$). Before the administration of IL1, T_{co}s were 39.0 ± 0.1 and 38.9 ± 0.1°C (mean ± SEM) in the two groups of animals. T_{co}s taken at the end of the 6-h recording period were 40.7 ± 0.2°C after 20 ng IL1 and 41.4 ± 0.2°C after 40 ng IL1. The increases in T_{co} corresponded to those found in T_{br}.

IL1 increased SWS, reduced W, and almost completely suppressed REMS (Figs. 19, 20). These effects were pronounced in hours 1-3 and were also significant across the 6-h recording period (Friedman's test, significant for both doses and for each state of vigilance; $P < 0.05$). A tendency for increased slow wave amplitude during SWS in postinjection hour 1 was observed (Table 7), though these changes were not significant ($P < 0.07$ for 20 ng, Wilcoxon matched-pairs signed-ranks test). Both doses of IL1 significantly decreased the median duration of W episodes, though only data for 20 ng IL1 are presented (Table 7).

Interactions of IL1 and αMSH

When the rabbits received αMSH following 40 ng IL1, fever continued to develop, but was attenuated (Fig. 18 A, B). Following 40 ng IL1 + 0.1 μg αMSH, the T_{br} curve was parallel and significantly lower than the 40 ng IL1 T_{br} curve (Friedman's test across 6 h; $P < 0.01$). The 0.5 μg dose of αMSH also attenuated 40 ng IL1-induced fever across the 6-h recording period (Friedman's test; $P < 0.01$).

Administration of 0.5 μg αMSH after 20 ng IL1 effectively prevented fever for 1 h (Wilcoxon matched-pairs signed-ranks test, $P < 0.05$; Fig. 18C).

Thereafter, T_{br} rose gradually, reached a maximum temperature change of 0.8°C and remained lower than that induced by 20 ng IL1 throughout the recording period (Friedman's across 6 h; $P < 0.01$). The 5.0 μg dose of αMSH blocked IL1-induced fever for 2 h (Wilcoxon matched-pairs signed-ranks test, $P < 0.05$; Fig. 18D). After this period, T_{br} increased slowly to about 1.0°C above the baseline level by the end of the recording, but still remained below that induced by 20 ng IL1 alone (Friedman's test across 6 h; $P < 0.01$). T_{co}s were 39.0 ± 0.1°C prior to ICV injection of IL1 and 40.2 ± 0.2°C at the end of the 6-h recording for both αMSH doses used with this IL1 dose.

Although the large increases in SWS induced by 40 ng IL1 tended to be attenuated 2-3 h after injection of 0.1 μg αMSH, these effects were not significant (Fig. 19). The low dose of αMSH, however, significantly attenuated the REMS suppressive effect of 40 ng IL1 (Friedman's test across 6 h; $P < 0.05$). When the administration of 40 ng IL1 was followed by injection of 0.5 μg αMSH, SWS decreased and W increased significantly with respect to the values obtained after IL1 alone (Friedman's test across 6 h; $P < 0.01$ for both SWS and W), though the animals still slept more than after aCSF injection alone. These effects appeared in postinjection hour 2 and thereafter. The REMS suppression induced by 40 ng IL1 was not affected by 0.5 μg αMSH (Fig. 19).

Administration of αMSH (0.5 and 5.0 μg) significantly attenuated the SWS-promoting effect of 20 ng IL1 (Fig. 20). After 0.5 μg αMSH, the IL1-induced increase in SWS was abolished in postinjection hour 1. This dose also attenuated the suppression of REMS induced by 20 ng IL1 (Friedman's test across 6 h; $P < 0.05$). When administration of 20 ng IL1 was followed by

injection of 5 μg αMSH, increases in SWS and decreases in W could no longer be observed; the values normally obtained after aCSF were restored. The suppression of REMS, however, was more pronounced than after IL1 alone. In response to αMSH, the tendency for IL1-induced increases in EEG slow wave amplitude in SWS disappeared (Table 7). The 5 μg αMSH dose reversed the pattern of increased number and reduced duration of W episodes elicited by IL1 in postinjection hour 1 (Table 7).

Rabbits injected with 20 ng IL1 + 0.5 or 5.0 μg αMSH continued to exhibit stretching/yawning and sexual excitation (Fig. 17B, D, F; cumulative numbers). The mean number of stretching/yawning events were four and nine per rabbit after 20 ng IL1 + 0.5 and 5.0 μg αMSH, respectively; the mean frequency of episodes with sexual excitation was 10 per rabbit after 20 ng IL1 + 5.0 μg αMSH, whereas no sexual arousal was observed in animals injected with 20 ng IL1 + 0.5 μg αMSH. These values were not different from those obtained from rabbits injected with αMSH without IL1 (see above). As after αMSH alone, the time spent grooming tended to decrease (Table 7). Also, the minutes in which the animals were active (exploration, stretching/yawning, and sexual arousal) decreased, whereas minutes spent lying down increased significantly in response to a combination of 20 ng IL1 + 5.0 μg αMSH. Ingestion was abolished by the adminstration of 5.0 μg αMSH to IL1-pretreated rabbits, as noted in the animals without IL1 injections.

Experiment 6

Effects of GRF in rats

The circadian variations of T_{br}, motor activity, and sleep-wake activity after the administration of aCSF were characteristic of those of normal rats. Thus, high values of T_{br} and motor activity and a high percentage of W were observed at night, while relatively low values of T_{br}, motor activity, less W, and more SWS and REMS were observed during the day (Fig. 21). ICV injection of GRF promoted sleep and decreased motor activity at the beginning of the night. In the remainder of the dark period and in the following light period, motor activity and the percentages of the vigilance states were at the baseline levels. GRF had no effects on T_{br}. Thus, the circadian rhythms of T_{br}, motor activity, and sleep-wake activity remained undisturbed after GRF injections. Although behavioral activities were not quantified, no apparent abnormalities were observed at the doses used.

GRF-induced changes were dose-dependent. The lowest dose (0.01 nmol/kg) decreased W and increased both SWS and REMS in postinjection hour 1 (Fig. 21, A). The mean level of motor activity did not change, suggesting that the rats compensated with increased activity during the W epochs for the loss of activity due to the increased sleep time. After increasing the dose of GRF (0.1 nmol/kg) (Fig. 21, B), a significant suppression of motor activity was also observed. These changes were confined to postinjection hour 1. Following the highest dose (1.0 nmol/kg) (Fig. 21, C) of GRF, however, the effects on SWS, W, and motor activity were more prolonged, while REMS did not change significantly. Thus, the reduction of W and the increase in SWS were

significant for the total of the first 3-h postinjection period (hours 1 to 3; W: 68.5 ± 4.1% after aCSF and 58.1 ± 4.7% after GRF [$p < 0.05$]; and SWS: 23.5 ± 3.3% after aCSF and 33.7 ± 3.8% after GRF [$p < 0.05$]), and also for the second 3-h period (hours 4 to 6, Fig. 21, C). Similarly, though the changes in motor activity calculated for individual hours were not significant, the means for the first and second 3-h postinjection periods after GRF decreased significantly with respect to the baseline values after aCSF. As a result of the prolonged suppression of W and increases in SWS, the rats slept more during the 12-h dark period after GRF than during the baseline night (W: 69.1 ± 2.7% vs. 63.7 ± 2.2% [$p < 0.05$]; SWS: 24.7 ± 1.8% vs. 30.2 ± 2.2% [$p < 0.05$]; and REMS: 6.1 ± 1.0% vs. 6.1 ± 0.8% [not significant]).

EEG power density values in the lowest frequency ranges (0.4-2.0 Hz and 2.4-4.0 Hz) were enhanced (Fig. 22, A, B). The statistical significance of the changes calculated for 30- or 60-min periods were marginal ($p < 0.2$) after the low dose, while substantial increases in EEG slow wave activity (2.4-4.0 Hz) were observed after the high doses ($p < 0.05$) (Fig. 22, C). Enhancement of EEG slow wave activity developed gradually, reaching a peak 30 to 50 min after 0.01 and 0.1 nmol/kg GRF. After the highest GRF dose (1 nmol/kg), the greatest increases in EEG power density values were observed in postinjection hour 2.

Effects of GRF in rabbits

In rabbits, there was little variation in sleep states over the 6-h recording period after ICV administration of aCSF. GRF elicited dose-dependent increases in sleep (Fig. 23). Thus, GRF induced a significant

suppression of W and increases in SWS after each dose in postinjection hour 1. Durations of W and SWS were close to baseline levels in postinjection hour 2 after the lowest dose of GRF, while the effects of the larger doses tended to last longer. REMS increased significantly in postinjection hour 2 after each dose. When the entire 6-h recording period was considered (Table 8), GRF-enhanced SWS was also significant after all doses, and GRF-enhanced REMS was significant after the two higher doses.

After each dose of GRF, the average EEG delta wave amplitudes calculated for 1-h periods increased significantly in postinjection hour 1, while the amplitudes in the other frequency bands were not affected (Fig. 24). After control injections of aCSF, amplitudes of EEG slow wave activity were higher during SWS than during W or REMS (Table 9). When calculated with respect to the sleep-wake states, significant increases were found in the mean delta amplitudes in SWS in response to 0.1 and 1.0 nmol/kg GRf. EEG amplitudes in W and REMS periods did not change.

Although not statistically appraised, rabbit behavior remained normal after the administration of GRF. Animals continued to cycle through the various states of vigilance, and whendisturbed during sleep, they could be aroused. T_{br} changes coupled to vigilance states (88) were also unaffected by GRf.

DISCUSSION

Experiment 1

Monomeric MPs: It was previously established that the substitution of muramic acid in muramyl peptides with an internal 1,6 anhydro bond strongly

enhances the somnogenic effect of those MPs when administered ICV in rabbits (87). The data presented here demonstrate that another manipulation of the C-6 atom of murmic acid, the substitution with acetic acid in an ester bond, enhances SWS activity as compared to the unsubstituted compounds. The effect, however, was not as pronounced as observed with the 1,6 anhydro form of muramic acid. Considering these effects we emphasize that small substitutions on the C-6-atom of muramic acid greatly modify somnogenic activity. Both these variations of the unsubstituted NAM will lessen the polarity of the molecule. Additionally, the formation of the 1,6 anhydro bond will change the spatial conformation of the sugar ring dramatically (Dr. G. Barnickle, Merck AG, Darmstad, FRG, personal communication). Whether spatial arrangements and/or changes in polarity of the NAM are responsible for the variations in somnogenic effect, cannot yet be decided. Both these structural variations are known to occur in bacteria. Since the ester bond of the O-acetyl group is relatively weak, it is tempting to speculate that this site may be enzymatically altered in vivo to regulate SWS-promoting activity of MPs.

The inhibiting effect of the unsubstituted terminal amidation of SWS-enhancing activity of MPs confirms earlier findings of this substitution on other monomeric MPs (80). Even one of the most active substances tested so far, NAG-(1,6 anhydro)NAM-Ala-γGlu-Dap-Ala-Ala, which was active at 1 pmol, was completely inactive at a dose of 100 pmol after the amidation of the terminal carboxyl group.

Dimeric MPs: Dimeric MPs have so far not been tested for biological activity although they provide a considerable portion of the digestion of bacbacterial cell walls by lysozyme, for example. We had two different types of

dimers in our possession: dimers that were formed by a peptide linkage of two monomers and dimers formed by a glycosidic bond of the sugar parts of two monomers. Both types of linkages are found in bacteria and it can be speculated that they both might be results of the processing of bacterial cell walls in a mammalian organism, depending on the sugar chain length and degrees of cross linkage of the respective peptidoglycan.

Peptide linked MP dimers did not show somnogenic activity. However, as was found for monomers (87), a 1,6 anyhydro substitution of NAM rendered a dimer somnogenic at a high dose. A clear pattern of structural requirements for somnogenic activity of glycosidic-linked MP dimers is not apparent.

Experiment 2

Results presented here replicate previous work to the extent that it was well known that damage to the POA results in insomnia (150) and hyperthermia (125, 133). Further, current results agree with previous data obtained from several different species (reviewed 8, 24) showing that the integrity of the POA is not essential for the induction of fever. The purpose of this study was to build upon those observations to explore the possible involvement of the POA in MDP-induced somnogenic responses. The major new findings reported here are that animals with damage to the POA: a) retain their capacity to elaborate somnogenic responses to MDP, b) exhibit reduced amplitudes of EEG slow waves during SWS, and c) continue to show T_{br} changes that are tightly coupled to vigilance states.

It has generally been thought that the POA is the primary locus of the febrigenic actions of pyrogens, based on the observations that, after injections of endogenous pyrogens (e.g., IL1, interferon, or tumor necrosis factor) into the POA, rapidly developing fevers are observed (8, 9). In addition, there is much evidence implicating the POA in sleep regulation. However, other sites outside the POA have also been implicated in fever and sleep regulation. Thus, febrile responses can be elicited after local injection of IL1 into several areas posterior to the POA (10). Damage to the raphe nuclei alters pyrogenic response to MDP (101) and induces insomnia (reviewed 150). Warming of the posterior hypothalamus enhances EEG spindle and slow-wave activity (4). Systemic injections of exogenous pyrogen depress firing rates of warm-sensitive and augment those of cold-sensitive neurons in the midbrain (112) and medullary region (135). Finally, microinjection of MDP into the POA and more posterior regions was reported to enhance SWS in rabbits (45). These results coupled with current results strongly suggest that although the POA is intimately involved in sleep and fever regulation, its integrity is not necessary to elicit such responses in a carefully controlled experimental setting.

An aspect of sleep not previously measured after POA damage is the amplitudes of EEG delta waves during SWS. In normal animals, enhanced amplitudes are observed during recovery sleep after sleep deprivation (13, 121) and during SWS after administration of certain putative sleep-promoting substances, e.g., MDP (77). It has been postulated, therefore, that alterations in EEG slow-wave amplitude may reflect the intensity of SWS. Early after POA damage, delta wave EEG amplitudes during SWS are reduced (Table 2) and remain lower than normal even as sleep duration recovers. This latter finding supports the concept of the POA as important in the control of cortical sleep-

like EEG based on electrical (150, 151) or thermal stimulation (4) of this area. However, in response to MDP, animals with damage to the POA retain their capacity to exhibit EEG slow-wave amplitudes greater than those observed without treatment (Table 2). Thus, it is possible that extra-POA sites may also be involved in the regulation of this phenomenon, as is the case of fever and sleep regulation.

Many substances that have the capacity to elicit fever and sleep responses are also intimately involved in inflammation/immune responses, e.g., prostaglandins (40), IL1 (85), and muramyl peptides (36, 85). Indeed, microinjections, which cause small lesions, of nonpyrogenic control solutions into the POA (125) and other regions (169) and micropuncture of the POA (133) result in slowly developing fevers. Further, microinjections of nonsomnogenic control solutions into the POA and brainstem areas also result in small increases in SWS (169). Thus, substances produced during wound-healing/inflammation responses elicited directly by the lesion may complicate responses elicited after lesions. For example, astrocytes have the ability to synthesize the pyrogenic/somnogenic cytokines, IL1 (40) and interferons (91). Thus, it is possible that their synthesis and release is enhanced during the inflammation that occurs after a lesion. In turn, they may diffuse to adjacent undamaged areas, eliciting hyperthermia. Hence, our observation of maximal hyperthermia three days postlesion with partial recovery of normal body temperature by 14 days post-lesion may reflect the course of the inflammation response in addition to direct damage to areas involved in thermoregulation. Indeed, recovery of the region bordering such lesions takes about 14 days (141).

The POA is involved in the regulation of multiple physiological functions besides T_{co} and sleep, e.g., glucose, osmotic and hormonal regulation, etc. Ample evidence exists suggesting that these functions are regulated in a coordinated fashion, e.g., single POA neurons are sensitive to both osmotic stimuli and temperature changes (147). It is also obvious that at some level each of these physiological functions is separate from the other because different effector organs are often involved. Moreover, IL1-induced fever, acute-phase responses, and sleep are separable (130, 144). However, it is not obvious whether each of these functions has independent neural regulatory pathways. Indeed, it seems likely that each of these functions is regulated by multiple sets of neurons, located in different parts of the brain, perhaps organized in a hierarchical fashion. Further, these multiple sets of neurons probably possess partially overlapping sensitivites to a variety of neuromodulators involved in the regulation of each function since many, if not all, neuromodulators affect more than one physiological system. The POA may allow optimization of responses to such stimuli. If this is the case, then damage to this area may not disrupt the responsiveness of any single system (sleep or other) to a neuromodulator, only the coordination between them.

Experiment 3

These experiments demonstrate that rabbits with S. aureus infections exhibit marked time-dependent changes in sleep patterns. These changes were characterized by initial increases in the time spent in SWS, the amplitude of EEG slow waves during SWS, and the duration of individual bouts of SWS. Subsequent to these effects, the amount of time spent in SWS, the EEG amplitude during SWS, and the SWS bout length all decreased below baseline values.

Similar biphasic responses occur during "recovery" sleep subsequent to sleep deprivation (13, 121, 161). S. aureus infection also inhibits REMS for up to 42 hr after inoculation; similarly, decreases in REMS can be observed during recovery, after prolonged periods of sleep deprivation (13, 43, 65). In addition, hematological changes similar to those produced by S. aureus infection have been described following total sleep deprivation (56).

In contrast to the effects of viable S. aureus, inoculation with the same number of heat-killed organisms did not alter sleep patterns in rabbits. However, sleep patterns were altered after the administration of a hundred-fold higher dose of killed organisms. These changes were qualitatively similar to those of viable S. aureus, but occurred with a much shorter latency and duration. These differences in the time courses of sleep responses following killed or viable S. aureus inoculations are probably related to differences in host immune responses or in the availability of MPs. Injection of large doses of killed bacteria would immediately expose the animal to both a large antigenic load and a relatively high dose of MPs contained within cell wall peptidoglycan. Indeed, after injection of purified MPs, the time courses of sleep responses are similar to those observed after injection of killed S. aureus (77). In contrast, when live bacteria are administered, cellular division occurs in vivo, and bacterial numbers gradually increase. Inhibition of the in vivo multiplication of S. aureus with antibiotic (cephalothin) treatment attenuates the magnitude and duration of S. aureus-induced effects, but does not alter the time of onset, thus providing further indirect evidence that differences in sleep responses may be related to the course of the host immune response.

A potential relationship between infectious disease and sleep has previously been suggested on the basis of observations that several immune response modifiers, including MPs and IL1, are also potent somnogens (78, 79, 85). These substances alter SWS in several species by increasing the amount of time spent in SWS and by enhancing amplitudes of EEG slow waves during sleep and the duration of individual bouts of SWS (reviewed in 84, 85). MPs have also been reported to inhibit REMS, although this effect varies depending on the species tested and the dose administered (84, 101). These effects of MP administration are thus qualitatively similar to the effects we have observed following infection with viable S. aureus, a bacterial organism containing MPs in its cell wall (42).

Although mammalian organisms do not synthesize MPs de novo (69), it has been suggested that MPs may be obtained from exogenous sources and then perhaps be chemically modified in vivo (1, 63). Indeed, the passage of muramyl dipeptide from the intestinal lumen into the blood has been described (122), and several body tissues contain MPs (76, 172). Mammalian macrophages possess surface receptors for MPs (148, 149), contain the enzymes necessary to cleave MPs from bacterial cell walls (166), and process bacterial cell walls to produce and release somnogenically active substances of low molecular weight (62). The processing of bacterial cell walls by macrophages may be a normal daily occurrence, as well as an early event in the initiation and amplification of the immune response. It is therefore possible that MPs could play a role in mammalian physiology, particularly during periods of bacterial infection when MP availability would be high due to the presence of abnormally large numbers of infectious organisms.

MPs are known to induce the in vivo production of another putative somnogen, IL1 (78, 123, 129). Macrophages stimulated by bacteria and bacterial cell wall products are also known to release IL1 (16, 31). IL1 mediates many of the acute-phase reactions that accompany infectious disease, and elevated levels of IL1-like activity have been reported in the circulation of febrile patients with bacterial infections (17). Moreover, levels of IL1-like activity in both plasma and cerebrospinal fluid increase during sleep (97, 106), and may also increase during and after sleep deprivation (107). Sleep patterns characterized by increased SWS and decreased REMS, as well as increased plasma IL1-like activity, have also been reported in humans following prolonged exercise (18, 142). Such observations suggest that the in vivo release of IL1 may also play a role in the enhancement of sleep during states of infectious disease.

Relatively little research has been performed on sleep during states of infectious disease, despite the common subjective experiences of lassitude or sleepiness under such conditions. Studies in the literature have not addressed this question in a carefully controlled manner with standard infectious challenges. For example, in human infants, mild upper respiratory tract infections, which were associated with rhinitis but not with fever, did not alter sleep state proportions or total sleep time (48), and only 4 of 14 adults with fever due to a variety of medical conditions demonstrated normal EEGs and enhanced SWS (92). Our data demonstrate that sleep is sequentially enhanced and suppressed during bacterial disease in rabbits, and thereby indicate that the time at which sleep is evaluated relative to the time of infectious challenge is a crucial consideration for the detection of consistent changes in sleep patterns during illness. The severity of the disease process

may also influence the type of sleep changes that occur. Abnormal EEG patterns have been associated with a number of experimental infectious encephalitides (3, 50, 49); however, in these studies, the central nervous system inflammation and damage associated with the infectious challenge are likely to have been responsible for the abnormal EEG activity that was observed. Indeed, historically, the study of CNS lesions produced during viral infections led Economo to describe sleep as an active process mediated by specific brain regions (34). Our results indicate that increased SWS accompanies the early stages of an infectious challenge, with a pattern resembling that observed during the recovery sleep that follows sleep deprivation. Furthermore, our observations of different patterns of sleep in animals that develop neutrophilia and successfully respond to the bacterial challenge, as compared to patterns observed in animals that become neutropenic and eventually succumb to the infection, suggest that sleep may provide a prognostic indicator under some conditions, and further imply that sleep may serve an adaptive function in combating infectious disease.

Experiment 4

The somnogenic actions of E. coli endotoxin and lipid A derived from S. typhimurium have been previously described (83). The present study expands upon these results by showing that different structural analogs of lipid A differentially affect sleep, EEG slow-wave activity, and T_{co}/T_{br}. In general, the results presented here concerning the structure of lipid-A molecules and their sleep/temperature effects parallel previous studies in which other biological activities of these substances were examined.

Compound 15-PP was the most active somnogenic and pyrogenic analog, and it produced these effects at lower doses than did any of the other compounds tested. Indeed, analog 15-PP was previously shown to possess full endotoxic activities and to have strong influences on pyrogenicity, leukopenia, chick embryo toxicity, Schwartzman reactions, immunoadjuvancy, and macrophage stimulation (80). Compound 15-PP is the substance corresponding to E. coli Re-mutant F515 lipid A. This hexaacyl diphosphoral lipid-A configuration has been described as the minimum structure which expresses the full range of typical endotoxic effects (74, 95, 128), and it is the most potent lipid-A structure tested thus far for inducing sleep.

The two monophosphoryl analogs tested here (compounds 15-PH and 16-PH) exhibited effects similar to, but weaker than, those of compound 15-PP in terms of SWS enhancement, REMS reduction, and pyrogenicity. Monophosphoryl lipid A analogs exert less of an effect on other biological/immunological activities (e.g., chicken embryo lethality, pyrogenicity, immunoadjuvant activity, and induction of tumor necrosis factor, interferon, and IL1) than do the corresponding diphosphoryl compounds (55, 67, 73). Thus, it could be predicted that diphosphoryl analogs would exert stronger sleep/temperature effects than would monophosphoryl compounds, as was previously described for lipid-A compounds derived from Salmonella typhimurium (83). Current results support this hypothesis in that compounds 18-PP and 15-PP, both diphosphoryl lipid A's, were somnogenic, and compound 16-PH, a monophosphoryl lipid A, had little activity. In contrast, compound 15-PH, the monophosphoryl analog of compound 15-PP, was somnogenic, although it was less effective than compound 15-PP.

Additional structural differences among the four lipid-A compounds tested here (besides the number of phosphate groups) that may contribute to variations in biological activation are the number, structure, and position of acyl residues on the glucosamine "backbone" of the lipid-A molecule. Both analogs 15-PP and 15-PH have the optimal acyl group configuration for endotoxic activity (two acyloxyacyl groups on C-2′ and C-3′), while 18-PP is tetraacyl with no acyloxyacyl residues (Fig. 11), partially explaining its low activity. Analog 16-PH is a monophosphoryl derivative of the parent septaacyl diphosphoryl molecule (compound LA-16-PP, derived from Salmonella minnesota; not tested here). The seventh acyl chain on 16-PH comes from an acyloxyacyl group at C-1, rather than a hydroxyacyl as is found on compounds 15-PP and 15-PH. The additional C-1 acyloxyacyl group lowers bioactivity (67, 74), and compound 16-PH was also found here to be less active in terms of sleep/temperature effects. Additional evidence indicating that acyl residues are important for biological activity stems from experiments in which lipopolysaccharide was treated with naturally occurring enzymes that cleave nonhydroxylated acyl chains but leave the rest of the molecule intact. After such treatment, a loss of bioactivity directly related to the degree of deacylation (acyloxyacyl removal) and reduced tissue toxicity was observed (108). The somnogenic activities of the compounds tested here further support the hypothesis that hexaacyl lipid-A molecules with acyloxyacyl chains on C-2′ and C-3′ but not on C-1 have generally more biological activity. Thus, the number, structure, and position of acyl residues, as well as the presence of phosphate groups, influence the bioactivity of different lipid-A molecules.

Naturally occurring lipid-A compounds enhance the synthesis and release of IL1 (29, 95), as well as that of tumor necrosis factor and interferons (55, 73, 74). These lymphokines are also somnogenic (86, 145) and probably

participate in a cascade of events leading, by one or more pathways, to the induction of sleep (145). Hexaacyl lipid-A molecules are more potent stimulators of IL1 (74, 95), interferon α/β (55, 73, 74), and tumor necrosis factor (55, 67, 73, 74). The corresponding monophosphoryl analogs (i.e., 15-PH, 16-PH) were poorer inducers of these lymphokines. The less toxic lipid-A precursor, 18-PP, has also displayed the ability to induce tumor necrosis factor and interferon, but to a lesser degree than the hexaacyl analogs (67, 73). Analog 16-PH, derived from S. minnesota, is a weak tumor necrosis factor-inducer and is unable to stimulate interferon (73). Thus, it is possible that lipid A and other similar compounds exert their different effects on sleep because of their differential abilities to influence cytokine production.

Lipid X is reported to be essentially nontoxic in relation to other disaccharide lipid-A synthetic analogs and natural lipid A (72, 157) and 10^5 times less pyrogenic (53). However, 100 ng of lipid X injected ICV increased SWS and REMS slightly, but did not significantly alter T_{co}. This effect may be related to the ability of lipid X to increase serum levels of tumor necrosis factor (54). However, tumor necrosis factor itself is pyrogenic (145) and has the capacity to induce IL1 production. Thus, the possibility exists that some other yet undefined mechanisms may be involved in the somnogenic actions of lipid X and the other substances tested here.

Experiment 5

ICV injection of αMSH induced reductions in T_{br}, REMS and SWS increases in W, specific behavioral patterns, and reduced somnogenic and febrile responses to IL1. The dose-dependent hypothermic effect of αMSH is a well-

established phenomenon in both rabbits (94) and guinea pigs (66). αMSH also reduces body temperature in squirrel monkeys (93), whereas it is without effect on thermoregulation in cats (126). The reductions in T_{br} reported here were similar to decreases in T_{co} observed in rabbits after comparable ICV αMSH doses. Lipton et al. (93, 94) report, however, that fever also may be attenuated with small doses of αMSH (usually around 0.2-0.25 μg ICV, though considerable individual variation exists) that are not hypothermic in afebrile animals. This suggests that the antipyretic mechanism is different from the hypothermic mechanism functioning after large αMSH doses. In the present experiments, even doses as low as 0.1 μg caused a slight and persistent downward shift in T_{br}. When the same dose of αMSH was combined with 40 ng IL1 in another group of animals, the persistent downward shift in the T_{br} curve was also evident. Further, when the challenge presented by IL1-induced fever was strong (40 ng IL1), even 0.5 μg αMSH produced only a persistent downward shift in the T_{br} curve, though more pronounced than the effect of 0.1 μg. It seems, therefore, that the small dose of αMSH either slightly reset the body thermostat or elicited a response too weak to require regulatory correction. αMSH concentrations in various rat brain regions exhibit diurnal variation, suggesting a role of αMSH in the circadian rhythms of behavioral, endocrine, and thermoregulatory functions (119). If endogenous αMSH, e.g., in the preoptic region, is involved in diurnal changes of body temperature, the effect of the small exogenous dose of αMSH we administered might be attributed to a promotion of this mechanism. At variance with this assumption, however, no changes in body temperature were reported following ICV administration of αMSH antiserum in rabbits (143).

The sensitivities of SWS and REMS to αMSH were slightly different. The threshold dose for reducing SWS, 0.5 μg αMSH, did not significantly affect REMS. More importantly, this dose of αMSH not only reduced the enhancement of SWS and the hyperthermia induced by 20 ng IL1, but also restored, at least partly, REMS. One explanation for these findings is that REMS suppression following IL1 may be at least partly regulatory; i.e., the intense functioning of SWS mechanisms inhibits the occurrence of REMS (90). Thus, by attenuating the SWS pressure induced by IL1, small doses of αMSH may facilitate an increase or partial recovery of REMS. Further, fever itself may inhibit REMS. αMSH reduces fever and thereby promotes the maintenance of optimum conditions for REMS. Higher doses of αMSH, however, inhibit REMS. Accordingly, when a high dose of αMSH (5.0 μg) was administered in combination with 20 ng IL1, REMS remained low, though the effects of IL1 on SWS and T_{br} were abolished.

Various observations suggest that αMSH may in fact increase W. Enhancement of learning and/or memory have repeatedly been reported following injection of αMSH (see 27 and 118 for reviews). Explanations for these findings vary greatly; however, increases in arousal or attention are common in most of them. In man, intravenous injection of $ACTH_{4-10}$, a fragment included in αMSH, elicited EEG signs of increased attention (105). Sandman et al. (136) report that systemic administration of αMSH in rats induces EEG activity characteristic of arousal. Chastrette and Cespuglio (20) did not observe increased W following ICV injection of αMSH in rats, possibly because the highest dose they used (0.1 μg) was too low. Concu et al. (23) found increased W and reduced SWS and REMS over an 8-h recording period after ICV administration of 25 μg ACTH in cats. Finally, the results of Olivo et al. (120) indicate that damage of αMSH/ACTH-containing neurons in the arcuate nucleus of newborn rats

by monosodium glutamate treatment causes a permanent reduction in W. Although the action of monosodium glutamate is not specific to opiomelanocortinergic neurons, this finding, as well as the others mentioned above, may indicate a role for endogenous, intraneuronal αMSH/ACTH in normal waking mechanisms.

Our behavioral studies confirmed that αMSH elicits stretching and yawning and sexual excitation as previously reported (6), whereas increases in grooming activity described in rabbits and rats were not observed. The threshold dose for sexual excitation was higher than that for stretching and yawning, supporting the notion of a different central neuronal basis for these αMSH effects (6). In consideration of behavioral effects, it is possible that increased W after αMSH injection and, particularly, reduction of sleep in rabbits pretreated with IL1 resulted from behavioral activation. αMSH-induced stretching and yawning or sexual arousal episodes were short, most often less than 1 min. Frequent occurrence of these episodes could seriously disturb sleep and result in increased W. However, instead of an increase in the number of short W episodes, the number of relatively long (4-min) W episodes increased after αMSH. Further, the time spent behaviorally active (including stretching and yawning, sexual excitation, grooming, ingestion, and general exploration) did not increase after αMSH. In contrast, a tendency toward decreased activity was observed. In agreement with these results, no increases in motor activity were found in rats injected with αMSH in any but a few experiments where motor activity was recorded (see 27 and 118 for reviews). αMSH also reduced EEG slow wave activity in SWS, indicating direct inhibition of sleep processes unrelated to motor activity. In addition, it seems that the autonomic effects of central administration of αMSH do not provide an explanation for increased arousal. Intrahypothalamic injection of αMSH

elicits slight increases in blood pressure and heart rate in rats (32). The major autonomic action of αMSH is the reduction of body temperature. However, substances with hypothermic effects might even increase sleep (e.g., cholecystokinin [66]). Further, IL1-enhanced sleep also decreased after αMSH, though body temperature did not fall below the normal level. It has been shown that antipyretics (e.g., anisomycin) block IL1-induced fever without attenuating the IL1 effects on SWS (78). Therefore, the attenuation of the sleep effects of IL1 following αMSH cannot be attributed to the reduction of fever. In conclusion, αMSH seems to have a capacity to increase vigilance that cannot be accounted for by the behavioral and autonomic actions of the peptide.

Opiomelanocortinergic neurons innervate various parts of the brain (see 105 for a review). The effects observed after ICV administration of αMSH might result from a combination of αMSH actions in widely different brain areas where endogenous opiomelanocortinergic projections terminate. The septum (94) and preoptic region (39) are implicated in the antipyretic effect, the preoptic region and the diagonal band of Broca in sexual stimulation (6), hypothalamic areas surrounding the third ventricle in stretching and yawning (6), the rostral septum and the posterior thalamic region in delaying the extinction of conditioned avoidance responses (11), and the dorsal hippocampus in increased grooming activity after αMSH/ACTH injection in rats (22). Although widespread high-affinity binding sites for IL1 have recently been described in the brain (38), the localization of the structures mediating the various central effects of IL1 is not known. αMSH failed to displace IL1 from receptors in the CNS (38); therefore, the interaction between the two substances is probably not direct.

αMSH has been proposed as an endogenous antipyretic that is released in response to IL1 and functions to suppress IL1-induced fever (94). αMSH, therefore, might be part of the negative feedback loop regulating IL1 actions. Another opiomelanocortin-derived peptide, pituitary ACTH, has a decisive role in this feedback mechanism; ACTH secretion in response to IL1 stimulates the production and secretion of glucocorticoids that inhibit further IL1 action and production (see 98 for a review). The release of ACTH is mediated through the secretion of hypothalamic corticotropin-releasing factor (CRF) (98); i.e., IL1β acts predominantly and IL1α acts exclusively through CRF. Lipton and his coworkers (94) suggest that CRF not only stimulates pituitary ACTH secretion, but hypothalamic CRF-containing neurons may also communicate with opiomelanocortinergic neurons in the hypothalamus, inducing release of αMSH in the septum. This proposal is based upon observations that ICV (but not systemic) administration of CRF reduces fever, and CRF concentration in the paraventricular nucleus decreases while αMSH concentration in the septum increases during IL1-induced fever (94). Suppression of feeding and increased arousal after αMSH injections in our experiments are also effects reported for CRF (35, 47). Hypothalamic neurons containing IL1β-like immunoreactivity have been reported to innervate the paraventricular and arcuate nuclei, where many CRF and αMSH cell bodies are located, respectively (15). Whether IL1 in fact can activate via CRF release two separate opiomelanocortin mechanisms, pituitary ACTH for regulating peripheral actions and hypothalamic αMSH for regulating its central actions, remains to be determined. Nevertheless, current results clearly indicate that αMSH can antagonize IL1-induced febrile and somnogenic actions.

Experiment 6

Results presented here clearly show that GRF has the capacity to enhance sleep in both rats and rabbits. These findings are consistent with the previous reports of Ehlers et al. and Nistico et al., who showed that GRF-induced suppression of rat locomotor activity increased EEG slow wave activity (35, 114), and increased SWS in a 30-min postinjection period (35). The GRF doses used by Nistico et al. (114) were similar to or lower than those in our experiments. Only a relatively high dose of GRF (2 nmol per rat) was effective in the studies by Ehlers et al. (35); lower and higher doses failed to increase EEG slow wave activity. The differences between the reported effective doses may be due to the specific peptide samples used. The preparation used by Nistico et al. (114) was the same as that injected in the present study (human GRF[1-40]), while Ehlers et al. (35) possibly administered GRF(1-44) (see 170).

Proposed sleep factors often failed to elicit consistent sleep responses when tested in various laboratories in conditions and species different from the ones used originally. It is important, therefore, that the effects of GRF were similar in rabbits and rats. Nevertheless, the sleep-promoting actions of GRF were generally more pronounced in rabbits than in rats; i.e., the largest percent increases in SWS and the greatest increases in EEG slow wave activity and REMS across all doses were found in rabbits. It is noted, however, that due to the anatomical and possible GRF-metabolizing differences, the effective doses of GRF reaching the yet unknown site of actions may be different in rabbits and rats. Further, the diurnal organization of sleep-wake cycles is different in rabbits and rats. While rabbits have small

diurnal variations in sleep-wake activity (75), rats are nocturnal animals and sleep mostly during the day. Thus, timing the injections to dark onset in rats, while injecting GRF into rabbits during the day, may have had an impact on the GRF-induced responses. Nevertheless, that low doses of GRF increased sleep in both rabbits and rats under different experimental conditions indicates a high somnogenic potency for the peptide.

The enhancement of EEG slow wave activity induced by GRF in rabbits (Fig. 24) resulted from both an enhancement of SWS duration (Fig. 23) and amplitudes of EEG slow waves during SWS (Table 9). EEG slow waves with increased amplitudes also occur in recovery sleep after sleep deprivation (12, 121). Such enhanced EEG slow waves may be indicative of the activity level of endogenous sleep mechanisms stimulated by sleep-promoting substances accumulating during prolonged W (12). That GRF and certain other putative sleep-promoting substances, e.g., IL1 (86), elicit these high amplitude EEG slow waves may thus suggest that a more intense SWS, similar to that observed after sleep deprivation, is induced by these substances.

The effects of sleep deprivation on GRF release remain unknown. It is noted, however, that human GH plasma levels remain low during a night of sleep deprivation and tend to be higher than normal, during recovery sleep the next night (138). This finding may indicate that GRF release is not enhanced during sleep deprivation; thus, GRF may not be responsible for feelings of sleepiness during sleep deprivation. Instead, GRF may be released in an increased quantity at the termination of sleep deprivation, inducing enhancements of both sleep and GH secretion. However, regulation of GH secretion is complex, involving many substances and feedback loops at various levels (reviewed in

44, 160). It remains to be clarified, therefore, whether blood GH content mirrors changes in GRF levels at those sites of action where the sleep-promoting effects are produced. Pulsatile pituitary GH secretion is controlled by the hypothalamic peptides GRF and somatostatin (SOM). SOM inhibits both GRF and GH release. GRF stimulates SOM release simultaneously with GH secretion, thus activating a regulatory mechanism which modulates GRF effects on the pituitary. GH released from the pituitary inhibits its own secretion and causes further stimulation of SOM release. Thus, SOM and the rise of GH levels in the blood act as negative feedback mechanisms for the secretion and endocrine effects of GRF (see 44, 160 for reviews).

The endocrine responses, e.g., rise in plasma GH levels and release of SOM, may also be involved in sleep regulation. High doses of systemically injected GH appear to inhibit SWS (104), suggesting a negative feedback role for GH in sleep regulation as well. Further ICV infusions of SOM in rats (26) and systemic administration of GH in rats (153), cats (33), and humans (104) induce selective increases in REMS. It seems, therefore, that in addition to acting as a negative feedback signal for SWS, GH and SOM also promote the occurrence of REMS subsequent to SWS. Thus, the development of the sleep cycle may require the complete neuroendocrine machinery involved in GH regulation. The long latency of increases in REMS following injections of GRF in rabbits (Fig. 23) supports the notion that the effects of GRF on REMs were indirect.

Since GRF promotes sleep and also releases GH from the pituitary, it is a likely candidate for a hypothalamic factor linking sleep and GH secretion. GRF is located in two main neuronal pools in the hypothalamus, one in the

arcuate nucleus and one near the ventromedial nucleus (25, 140). The arcuate nucleus is probably the primary source of GRF-containing terminals innervating the median eminence and thereby regulating pituitary GH release. The function of the GRF-containing neurons around the ventromedial nucleus is less clear. Various lines of evidence indicate that these neurons might also be involved in the stimulation of GH release (44, 99), and, in fact, the existence of a few projections from these neurons to the median eminance was shown (25). GRF-containing neurons around the ventral medial nucleus, however, also innervate various parts of the basal forebrain, suggesting a neurotransmitter/neuromodulatory function for the peptide (25, 140). This notion has been supported by the demonstration of neurons responsive to local application of GRF in the basal forebrain and other parts of the CNS (163). The rostral part of the basal forebrain, i.e., an area extending from the anterior hypothalamus to the olfactory tubercle, has an important role in eliciting and maintaining SWS (reviewed in 152). Thus, the projections from the GRF-containing neurons around the ventromedial nucleus to the median eminence and basal forebrain structures may provide the morphological basis for the two separate outputs activating GH secretion and sleep mechanisms, respectively.

GRF belongs to a family of structurally related peptides which also includes vasoactive intestinal peptide (VIP), histidine/isoleucine-containing peptide (PHI), secretin, glucagon and gastric inhibitory peptide (GIP). The structural homologies between these peptides may explain, at least partially, the similarity of responses to their exogenous administration. For example, hypothalamic neurons often react the same way to both GRF and PHI (163), and ICV injection of VIP induces GH release (167), though endogenous VIP in the hypothalamus is probably not involved in the physiological regulation of GH

secretion. Most important within the context of current results is that VIP has sleep-promoting activity in rats similar to that of GRF (117). Thus, ICV injections of VIP also induce enhanced SWS and REMS without affecting thermoregulatory mechanisms. In contrast, other putative sleep-promoting substances we have investigated under similar experimental conditions used here induced responses distinct from those elicited by GRF and VIP. Cholecystokinin (CCK), injected intraperitoneally in rats at dark onset, promotes SWS and EEG slow wave activity but also induces hypothermia and does not affect REMS (68). Prostaglandin D_2 enhances SWS in both rats (164) and rabbits (82) but induces hypothermia in rats and hyperthermia in rabbits. Interleukin-1 has the capacity to enhance SWS and EEG slow wave activity for prolonged periods (4-10 hours depending on dose), but it inhibits REMS (78). Muramyl peptides (MPs) also enhance SWS and EEG slow wave amplitudes for several hours and either inhibit, enhance or have little effect on REMS depending upon dose, species, specific MP used, and the time of day injections are made (reviewed in 82, 88). Unlike GRF, somnogenic doses of IL1 and certain MPs induce fever, though temperature responses could be separated from sleep responses (82, 88). In our laboratories, DSIP did not promote sleep when administered ICV at night in rats (116) or during the day in rabbits (75). In contrast, some analogs of DSIP with increased resistance to enzymatic degradation increases sleep but only after long (several hours) latencies (116).

Several putative sleep factors (reviewed in 90) may share with GRF common regulatory pathways. Thus, CCK, PGs, IL1, insulin, and DSIP can alter GH secretion and/or other substances involved in GH regulation. For example, CCK releases VIP (159), delta sleep-inducing peptide (DSIP) inhibits SOM release (60), insulin has been commonly used to provoke GH release, and serotonin

(109), PGs (70), and IL1 (5) can also alter GH release under certain experimental conditions. Although it remains to be clarified whether the somnogenic actions of any or all of these sleep factors are mediated by stimulating GRF release or facilitating or mimicking GRF effects, it is tempting to speculate that at least some of the sleep-promoting substances are involved in a cascade of biochemical events, perhaps compartmentalized within the brain, that plays a role in sleep regulation.

GENERAL DISCUSSION

We emphasize that the somnogenic actions of MPs are probably mediated via some of the other SFs mentioned. We envision sleep regulation by multiple SFs in the following way. 1) Each SF will have defined receptors on neurons and/or glia that initiate another event when stimulated. 2) Only some of the SF-receptor interactions will be involved in the cascade of events regulating sleep. 3) The other SF-receptor interactions are involved in other biological activities of the SF. It is, therefore, theoretically possible both to separate these activities from sleep activity, although there is likely to be much overlap between different neuronal subset populations, and to affect sleep responses by manipulation of these subset populations by means other than SFs. 4) The "sleep" subset of one SF may, but does not necessarily, overlap with that of another SF, thus providing redundancy and integration. 5) It is likely that there are multiple endogenous antagonists of the SFs which could act at various levels, e.g., intracellular feedback regulation. Such a regulatory scheme is consistent with what we know about SFs, brain mechanisms of sleep, and other physiological regulatory systems.

Sleep regulatory mechanisms are likely to involve a series or cascade of discrete events including feedback loops which ultimately give rise to sleep. Further, for each substance involved in this regulatory pathway(s), there will be a metabolic (anabolic/catabolic) pathway that also involves multiple substances. It is, therefore, important to ask if there are interactions between SFs, for some, or many, may be part of the same regulatory scheme. Many interactions between putative SFs have been described (reviewed 89), although most have been described in vitro. Thus, it is difficult to know if they also occur in vivo in brain. In addition to direct interaction between SFs, it is also possible for a SF to affect a cellular component, which in turn may affect activity of another SF. For example, the expression in glia of class II major histocompatibility antigens such as HLA-DR2 can be enhanced by MPs; in turn, HLA-DR antigen can enhance cell sensitivity to IL1 (reviewed 85). To fully understand biochemical regulation of sleep, it is crucial to define biochemical pathways involved in sleep and how they are regulated. Each of the many substances involved in such pathways could be called a SF (or waking factor) because its introduction into an animal would be likely to modify sleep-wakefulness.

All endogenous SFs have probably not yet been identified nor have all the interactions between various known SFs been described. Nevertheless, much progress has occurred since the start of our contract period two years ago. We now recognize that MPs probably elicit their somnogenic effects via cytokines which, in turn, probably elicit their somnogenic effects via PGs and/or various neuroendocrines. Further, we now recognize that there are regulatory feedback loops for SFs which can alter SF production and production of SF receptors. Such developments have already greatly enhanced our understanding

of sleep regulation. Perhaps more important within the context of the general objectives of this contract, new possibilities have opened up concerning the development of new somnogenic agents. Over the remaining three years of this contract, we will begin to explore some of these possibilities.

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Table 1: Somnogenic and pyrogenic activities of various muramyl peptides

No.	Composition	MW/MH+	n	dose pmol	SWS(2-6h) C	SWS(2-6h) E	TRMS(2-6h) C	TRMS(2-6h) E	Temp (6h) C	Temp (6h) E
(1)	NAG-(6-O-Acetyl)NAM-Ala-γGlu-Dap	911	4	10	54±3	56±1	9.6±1.8	9.8±1.2	39.5±0.1	39.8±0.1
			4	100	54±3	57±3	9.6±1.8	9.3±1.1	39.5±0.1	40.1±0.2+
(2)	NAG-NAM-Ala-γGlu-Dap	869	3	1	43±6	43±9	6.9±0.6	4.4±1.3	39.2±0.4	39.4±0.3S
			4	10	47±3	50±3	7.7±1.5	9.5±2.2	39.1±0.2	39.6±0.2S
(3)	NAG-(6-O-Acetyl)NAM-Ala-γGlu-Dap-Ala	982	8	1	56±3	54±2	2.5±0.7	1.6±0.5	39.8±0.1	40.1±0.1+
			4	10	49±1	63±5*	7.1±0.5	4.2±0.9*	39.4±0.1	40.3±0.1+
(4)	NAG-NAM-Ala-γGlu-Dap-Ala	940	4	1	44±4	52±3	8.1±0.9	7.0±0.7	39.2±0.1	39.7±0.3S
			4	10	51±2	53±3	7.3±2.1	6.4±2.1	39.2±0.0	40.3±0.2S
			4	100	46±2	55±3*	2.5±0.3	3.8±1.1	39.5±0.0	40.2±0.3+
(5)	NAG-(6-O-Acetyl)NAM-Ala-γGlu-Dap-Ala-Ala	1053	4	1	50±3	51±2	7.5±0.9	5.9±1.3	39.5±0.1	39.7±0.2
			4	10	42±2	54±4*	9.9±0.3	7.5±1.0	39.4±0.0	40.8±0.1+
			4	100	42±2	70±4*	9.9±0.3	4.8±0.6*	39.4±0.0	41.7±0.1+
(6)	NAG-NAM-Ala-γGlu-Dap-Ala-Ala	1011	2	1	40,46	38,49	9.5,16.1	6.7,7.5	39.1,39.2	39.5,39.6S
			4	10	51±3	57±2	7.4±1.7	6.1±0.9	39.3±0.1	40.0±0.3S
(7)	NAG-(1,6-anhydro)NAM-Ala-γGlu-Dap-Ala-AlaNH_2	920	4	10	41±1	41±2	3.4±0.9	3.3±0.7	39.4±0.1	39.3±0.1
			4	100	56±2	57±4	4.8±1.1	4.7±1.2	39.4±0.0	40.0±0.1+
(8)	NAG-NAM-Ala-γGlu-Dap-Ala-AlaNH_2	938	3	10	50±3	56±3	3.5±1.3	3.9±1.4	39.3±0.1	39.3±0.1
			4	100	57±2	61±5	5.7±0.6	3.3±1.1	39.2±0.1	39.7±0.2
(9)	NAG-NAM-Ala-γGlu-Dap-Ala	1860	4	10	52±3	57±2	2.7±0.9	5.2±1.4*	39.3±0.1	39.3±0.1
			4	100	47±3	49±5	3.2±1.2	4.2±0.6*	39.3±0.0	39.3±0.1
	NAG-NAM-Ala-γGlu-Dap-Ala									
(10)	NAG-NAM-Ala-γGlu-Dap-Ala-Ala	1931	4	10	57±4	56±6	3.6±1.0	3.3±1.0	39.2±0.2	39.2±0.3
			4	100	49±1	50±2	7.1±0.5	3.3±0.2*	39.4±0.1	39.7±0.2
	NAG-NAM-Ala-γGlu-Dap-Ala									
(11)	NAG-(1,6-anhydro)NAM-Ala-γGlu-Dap-Ala	1842	4	10	53±4	49±2	4.6±0.5	3.1±0.4	39.2±0.1	39.7±0.2+
			4	100	51±3	62±3*	3.6±1.3	4.2±1.2	39.4±0.1	40.9±0.1+
	NAG-NAM-Ala-γGlu-Dap-Ala									
(12)	NAG-(1,6-anhydro)NAM-Ala-γGlu-Dap-AlaNH_2	1840	4	10	45±2	48±2	2.5±0.7	3.5±0.9	39.3±0.0	39.5±0.1
			4	100	53±4	53±2	4.6±0.5	2.2±0.8	39.2±0.1	39.6±0.1
	NAG-NAM-Ala-γGlu-Dap-AlaNH_2									

(Table 1 continued)

No.	Composition	MW/MH+	n	dose pmol	ISWS(2-6h) C	ISWS(2-6h) E	IREMS(2-6h) C	IREMS(2-6h) E	Temp (6h) C	Temp (6h) E
(13)	NAG-NAM-Ala-γGlu-Dap-AlaNH$_2$	1858	4	10	52±3	45±2	3.6±1.1	4.5±1.7	39.1±0.1	39.5±0.1
			4	100	51±3	53±4	4.4±0.6	2.6±1.0	39.1±0.0	39.5±0.0
	NAG-NAM-Ala-γGlu-Dap-AlaNH$_2$									
(14)	NAG-NAM-Ala-γGluNH$_2$	1414	4	10	49±1	55±3*	7.1±0.5	4.4±1.1*	39.4±0.1	39.8±0.1
			4	100	51±2	62±6*	6.1±1.0	4.0±1.3	39.3±0.2	40.3±0.3+
	NAG-(6-O-acetyl)NAM-Ala-γGluNH$_2$									
(15)	NAG-NAM-Ala-γGluNH$_2$	1372	4	100	43±3	61±3*	4.2±0.9	3.5±0.6	39.3±0.1	40.7±0.1+
	NAG-NAM-Ala-γGluNH$_2$									
(16)	NAG-NAM-L-Ala-D-iGln	(764)	4	100	49±3	65±6*	7.9±0.9	1.9±0.4*	39.2±0.1	41.1±0.0+
(17)	NAG-NAM	(496)	4	100	52±2	51±1	8.7±1.4	9.6±1.7	39.5±0.1	39.5±0.1
(18)	NAG-NAM-L-Ala-D-iGln-I-Ala-D-iGln	(1093)	4	100	52±2	52±2	8.7±1.4	10.5±1.5	39.5±0.1	39.5±0.1
(19)	NAG-NAM-NAG-NAM-L-Ala-D-iGln-L-Ala-D-iGln	(1372)	4	100	53±2	61±3	9.9±2.0	9.0±1.5	39.0±0.2	39.6±0.3

+ = significant increase in temperature (> 0.5 °C)
* = significant difference in Friedman test
$ = from reference 87

Table 2. Effects of preoptic area lesions on rabbit sleep and temperature.

			Day 2	Day 3	Day > 8	Day > 9
	Prelesion		Postlesion		Postlesion	
	NI[a]	MDP[a]	NI[a]	MDP[a]	NI[b]	MDP[b]
% SWS	53 ± 2	70 ± 3*	24 ± 3†	46 ± 5*†	43 ± 3	52 ± 2*
No. SWS episodes[c]	103 ± 6	140 ± 9*	88 ± 11	182 ± 17*	148 ± 12	172 ± 15
Duration of SWS episodes[d]	1.9 ± 0.2	1.9 ± 0.2	1.0 ± 0.1†	1.0 ± 0.1†	0.8 ± 0.1†	1.4 ± 0.2*
EEG amplitude[e]	177 ± 13	235 ± 20*	132 ± 9†	160 ± 15†	109 ± 5†	172 ± 14*
% REM[f]	8.4 ± 0.8	1.6 ± 0.6*	0.9 ± 0.3†	0.2 ± 0.1†	2.2 ± 0.8†	0.7 ± 0.2*
Colonic Temp.[g]	39.1 ± 0.05	40.6 ± 0.2*	40.5 ± 0.2†	41.5 ± 0.2*†	39.8 ± 0.2†	40.7 ± 0.2*

[a]n = 12; paired t-tests used; all animals same as prelesion animals; NI is no infusion.

[b]n = 8; paired t-test used; all animals were part of prelesion group.

[c]Number of episodes of 6-h recording period.

[d]Duration of SWS episodes in min; minimum duration was 0.2 min because records were scored in 12-s epochs.

[e]EEG amplitudes in the 0.5-4.0 Hz frequency bands during periods of SWS; values in μV.

[f]Number and duration of REM sleep episodes were not determined since many animals (n = 6) did not have REM sleep after the lesion nor after MDP treatment (n = 9).

[g]Colonic temperatures taken 5 h after beginning of recording.

*Indicates significantly different from corresponding NI value.

†Indicates significantly different from corresponding prelesion values.

Table 3

Effects of Staphylococcus aureus Inoculation on Plasma Cortisol Levels

	Time postinoculation (hr)			
S. aureus	0+	6	12	24
Viable (10^7 to 10^8 CFU) (n = 16)	3.3 ± 0.3	10.3 ± 0.9*	13.5 ± 1.1*	12.2 ± 3.0*
Killed (7×10^9 CFU) (n = 12)	3.5 ± 0.5	10.7 ± 1.6*	10.0 ± 1.4*	9.9 ± 2.3*
Killed (8×10^7 CFU) (n = 8)	2.6 ± 0.2	5.9 ± 0.8*	5.2 ± 0.8*	2.9 ± 0.2

Values represent μg/dl of plasma cortisol.

+Time 0 samples were taken just before inoculation with S. aureus.

*$p < 0.01$ relative to time 0.

Table 4

Comparison of Sleep Patterns in Animals that Died from or Survived Inoculation with Viable Staphylococcus aureus

	Time postinoculation (hr)					
	2	4	6	8	10	12
% Time in SWS						
Group I	38 ± 5	56 ± 6	66 ± 6	61 ± 8	41 ± 12	34 ± 8
Group II	36 ± 3	54 ± 3	59 ± 6	70 ± 4	75 ± 4*	70 ± 5*
Slow Wave Amplitude (% control)						
Group I	102 ± 1	108 ± 2	110 ± 7	100 ± 4	86 ± 5	81 ± 3
Group II	104 ± 2	110 ± 1	112 ± 2	118 ± 2*	109 ± 2*	103 ± 2*
SWS Bout Length (min)						
Group I	3.8 ± 0.3	5.5 ± 0.9	6.7 ± 1.0	5.0 ± 0.8	3.0 ± 0.7	3.2 ± 0.5
Group II	4.3 ± 0.3	6.1 ± 0.7	6.5 ± 0.5	13.2 ± 3.0*	11.1 ± 1.2*	15.0 ± 6.7*

*Significant differences between animals that died (Group I; n = 6) and animals that survived (Group II; n = 18); $p < 0.01$.

Table 5

Comparison of Clinical Indices in Animals that Died from or Survived Inoculation with Viable Staphylococcus aureus

	Time postinoculation (hr)		
	0	6	12
Temperature (°C)			
Group I	39.0 ± 0.2	40.5 ± 0.3	40.2 ± 0.4
Group II	38.9 ± 0.1	40.0 ± 0.1*	40.7 ± 0.1*
Cortisol (μg/dl)			
Group I	3.0 ± 0.1	13.5 ± 1.3	16.6 ± 1.3
Group II	3.6 ± 0.5	9.4 ± 0.8*	13.1 ± 1.2
Neutrophils (% control)			
Group I	100	130 ± 22	88 ± 25
Group II	100	204 ± 214*	211 ± 26*
Lymphocytes (% control)			
Group I	100	42 ± 6	13 ± 3
Group II	100	42 ± 4	18 ± 3
nRBCs (#/100 WBCs)			
Group I	0.3 ± 0.2	2.0 ± 1.4	22.5 ± 12.2
Group II	0.8 ± 0.3	0.5 ± 0.2	1.5 ± 0.3*

*Significant difference between animals that died (Group I, n = 6) and animals that survived (Group II, n = 18); $p < 0.03$.

TABLE 6. Effects of lipid-A compounds and lipid-X on rabbit slow-wave sleep (SWS), rapid-eye-movement sleep (REMS), electroencephalographic delta voltages, and 6-hr colonic temperatures.

Substance	Inject. Route	Dose	n	Percent SWS		Percent REMS		Delta Voltages		Colonic Temperature, °C	
				Control	Expt.	Control	Expt.	Control	Expt.	Control	Expt.
506	iv[a]	0.03 μg/kg	4	48 ± 4	62 ± 4[c]	4.6 ± 0.8	2.5 ± 1.5	± 3	86 ± 15[c]	38.9	39.3
	iv	0.3 μg/kg	4	56 ± 3	83 ± 3[c]	4.6 ± 0.8	0.1 ± 0.1[c]	75 ± 8	120 ± 16[c]	39.0	39.6[d]
	icv[b]	1 ng	4	36 ± 2	42 ± 4[c]	4.8 ± 0.5	5.7 ± 0.6	75 ± 7	88 ± 6[c]	39.3	39.7[d]
	icv	10 ng	4	44 ± 2	46 ± 3	5.8 ± 0.8	3.3 ± 0.7	113 ± 8	126 ± 11[c]	39.2	40.4[d]
504	iv	0.3 μg/kg	4	50 ± 3	50 ± 1	6.0 ± 0.4	2.1 ± 0.8[c]	73 ± 5	72 ± 5	39.2	39.3
	iv	3.3 μg/kg	8	53 ± 1	65 ± 2[c]	3.8 ± 0.8	1.1 ± 0.4[c]	104 ± 9	121 ± 12[c]	39.3	39.9[d]
	icv	1 ng	4	55 ± 4	54 ± 2	3.4 ± 0.9	2.0 ± 0.4	107 ± 2	113 ± 6	39.5	39.6
	icv	10 ng	8	50 ± 3	53 ± 2	4.2 ± 1.5	2.6 ± 0.6	na	na	39.5	40.3[d]
LA-18-PP	iv	0.3 μg/kg	4	38 ± 2	41 ± 3	4.9 ± 0.8	4.7 ± 0.9	93 ± 6	102 ± 11	39.1	39.3
	iv	3.3 μg/kg	4	38 ± 2	53 ± 2[c]	4.8 ± 0.5	3.6 ± 0.5	75 ± 7	99 ± 8[c]	39.3	39.3
	icv	1 ng	4	44 ± 2	41 ± 2	5.8 ± 0.8	5.5 ± 0.3	113 ± 8	111 ± 6	39.1	39.4
	icv	10 ng	8	39 ± 2	42 ± 3	4.9 ± 0.3	4.8 ± 0.6	81 ± 4	90 ± 4[c]	39.3	40.1[d]
514	iv	0.3 μg/kg	4	50 ± 3	52 ± 4	6.3 ± 1.2	3.1 ± 0.7[c]	97 ± 5	101 ± 7	39.3	39.4
	iv	3.3 μg/kg	4	55 ± 4	49 ± 2	3.6 ± 0.8	3.0 ± 0.9	107 ± 2	115 ± 4[c]	39.5	39.5
	iv	33.0 μg/kg	4	48 ± 4	45 ± 2	3.0 ± 0.9	2.3 ± 0.3	79 ± 11	76 ± 8	39.6	39.5
	icv	1 ng	3	5	52 ± 4	4.9 ± 0.3	2.5 ± 0.9	82 ± 1	84 ± 10	39.1	39.4
	icv	10 ng	4	54 ± 2	52 ± 2	4.7 ± 1.0	2.4 ± 0.5	105 ± 16	105 ± 11	39.2	39.5
	icv	100 ng	4	52 ± 1	50 ± 2	1.8 ± 0.7	2.4 ± 0.7	78 ± 5	84 ± 7	39.2	39.1
Lipid-X	iv	3.3 μg/kg	4	38 ± 2	35 ± 3	4.7 ± 0.8	6.1 ± 0.6	85 ± 5	89 ± 5	39.5	39.2
	iv	33.0 μg/kg	4	41 ± 2	44 ± 3	6.1 ± 0.8	4.2 ± 0.4	131 ± 5	132 ± 9	39.2	39.2
	icv	10 ng	4	43 ± 2	38 ± 3	5.0 ± 0.3	4.6 ± 0.9	88 ± 2	91 ± 2	39.2	39.5
	icv	100 ng	4	38 ± 2	45 ± 2[c]	4.9 ± 0.8	6.6 ± 1.2[c]	85 ± 6	92 ± 4	39.1	39.3

[a] intravenous.

[b] intracerebroventricular.

[c] Wilcoxon matched pairs test, $p \leq 0.05$.

[d] temperature increased ≥ 0.5°C.

na = not available.

Data shown are means ± SE; all temperatures SE ≤ 0.1

Table 7. Effects of αMSH and 20 ng IL1 + αMSH on duration of W episodes, EEG slow wave amplitude, and behavior in rabbits during postinjection hour 1.

Manipulation	N	Number of W Episodes in Postinjection Hour 1	Median Duration of W Episodes (min)	Mean (± SEM) Slow Wave Amplitudes during NREMS (μV)	N	Time Spent in Behavior (min/h)			
						Inactive	Active	Grooming	Ingestion
0.1 μg αMSH	3	88	0.8	272.8 ± 24.1					
aCSF		87	0.8	298.6 ± 22.9					
0.5 μg αMSH	6	107	1.6*	242.8 ± 9.1+	6	47.6 ± 2.5+	14.3 ± 2.4	4.7 ± 1.2	2.3 ± 1.2
aCSF		73	0.4	293.4 ± 11.0		38.6 ± 3.0	9.2 ± 1.8	2.5 ± 1.2	0.7 ± 0.6
5.0 μg αMSH	8	117	1.2*	294.3 ± 19.4+	8	49.6 ± 1.6	9.9 ± 1.8	1.7 ± 0.6	0+
aCSF		76	0.4	354.8 ± 19.4		44.4 ± 2.3	8.5 ± 1.8	3.8 ± 1.2	2.0 ± 0.6
50.0 μg αMSH	7	64	2.0*	243.8 ± 12.9+					
aCSF		48	0.8	302.5 ± 4.6					
20 ng IL1	8	142+	0.4*	328.5 ± 14.9					
aCSF		85	0.8	292.6 ± 12.4					
20 ng IL1 + 0.5 μg αMSH	8	124	0.4	309.6 ± 14.6	6	43.5 ± 2.9	10.5 ± 1.8	4.3 ± 1.8	1.7 ± 1.2
aCSF						38.3 ± 1.8	12.8 ± 1.8	6.2 ± 1.2	2.6 ± 1.2
20 ng IL1 + 5.0 μg αMSH	8	75†+	1.2†*	260.0 ± 14.5	8	48.9 ± 1.3+	8.0 ± 1.2+	3.1 ± 1.2	0+
aCSF						37.9 ± 2.7	13.9 ± 1.9	3.8 ± 0.8	4.4 ± 1.0

*$P < 0.05$, medians test

+$P < 0.05$, Wilcoxon matched-pairs signed-ranks test

†+, †*$P < 0.05$, compared to 20 ng IL1

Table 8. Changes in the vigilance states in response to icv administration of various doses of GRF in rabbits.

	N	W†	NREMS†	REMS†
GRF 0.01 nmol/kg	7	- 5.8 ± 2.3*	+ 4.8 ± 1.8*	+1.0 ± 0.6
GRF 0.1 nmol/kg	8	-10.9 ± 1.5*	+ 7.9 ± 1.3*	+3.0 ± 1.1*
GRF 1.0 nmol/kg	6	-19.8 ± 3.3*	+14.8 ± 3.2*	+5.0 ± 0.7*

*Asterisks denote significant differences from baseline (Wilcoxon matched pairs signed ranks test, $p < 0.05$).

†Values are means ± SE of experimental-control percentages of time spent in each state during the 6-h recording period.

Table 9. Average voltages of EEG slow waves (0.5-3.5 Hz) after icv injection of aCSF or various doses of GRF in rabbits.

		Wakefulness†		NREMS†		REMS†	
Dose	N	aCSF	GRF	aCSF	GRF	aCSF	GRF
0.01 nmol/kg	7	46.3 ± 3.8	49.3 ± 2.9	144.7 ± 14.8	165.9 ± 14.2	43.9 ± 3.5	44.2 ± 2.9
0.1 nmol/kg	8	42.9 ± 3.8	44.9 ± 3.7	127.3 ± 17.2	159.5 ± 18.8*	35.7 ± 3.4	41.5 ± 4.0
1.0 nmol/kg	6	51.0 ± 3.9	59.7 ± 4.9	150.0 ± 16.2	186.4 ± 22.1*	42.4 ± 4.4	47.2 ± 4.4

*Asterisks denote significant differences with respect to baseline values (Wilcoxon matched pairs signed ranks test, $p < 0.05$).

†Values shown are average μV (means ± SE) obtained during vigilance states (W, NREMS, and REMS) during postinjection hour 1.

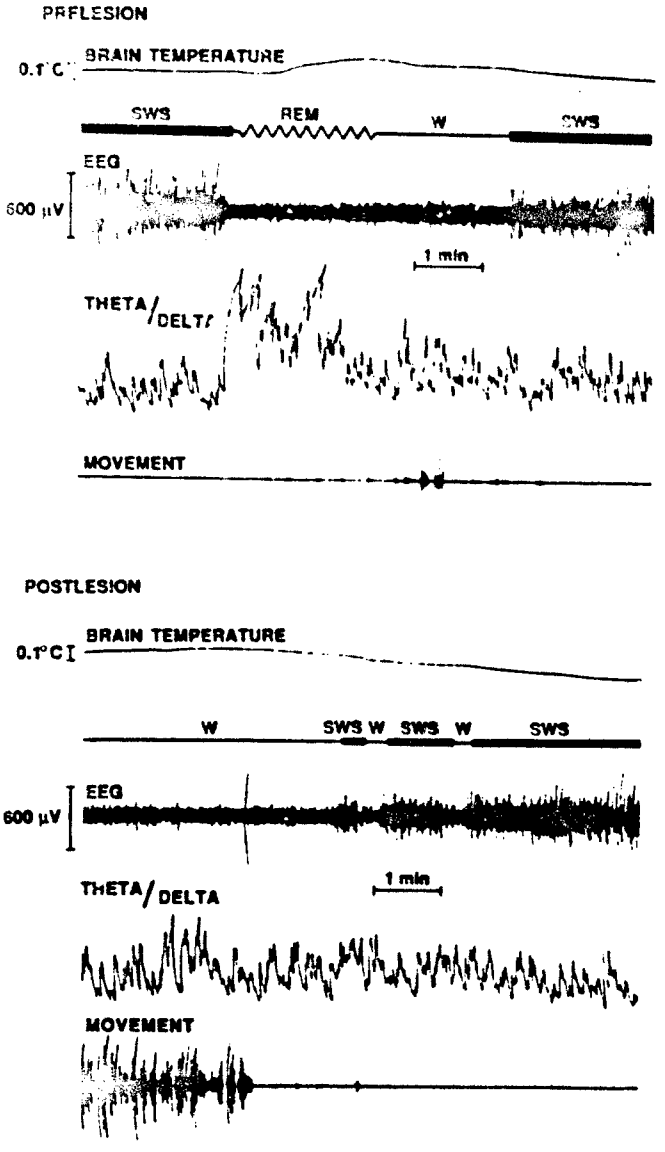


Fig. 1: Recordings from a rabbit before (A) and after (B) preoptic area (POA) lesions. In A and B, recording conditions were identical; i.e., the same electrode, cage, and amplifier gains were used. After the lesion (B), amplitudes of the EEG during SWS were lower than before the lesion (see Table 2). The criteria used to identify states of viligance in normal rabbits can be used for state identification after lesions.

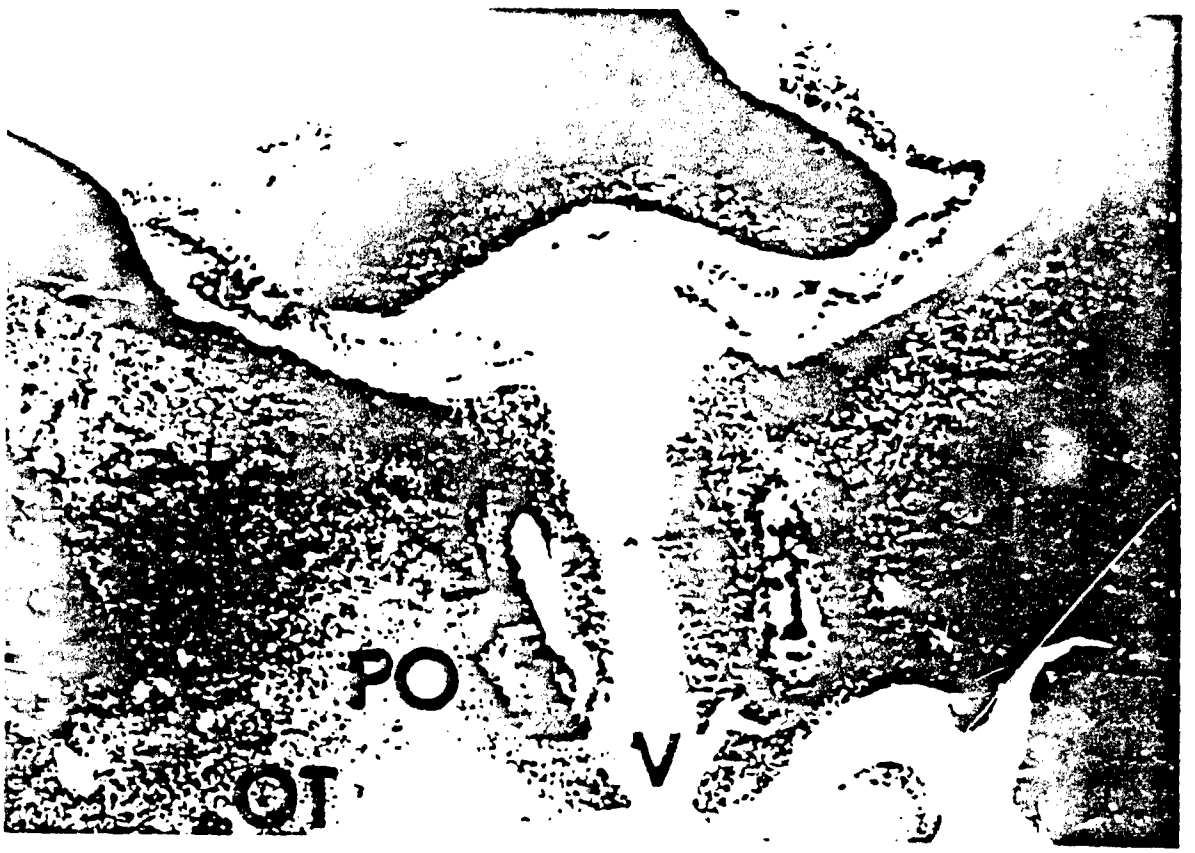


Fig. 2: Coronal section of a rabbit brain (165) at the level of the POA, showing the extent of the lesions observed in the majority of the animals. 3V = third ventricle; OC = optic chiasma.

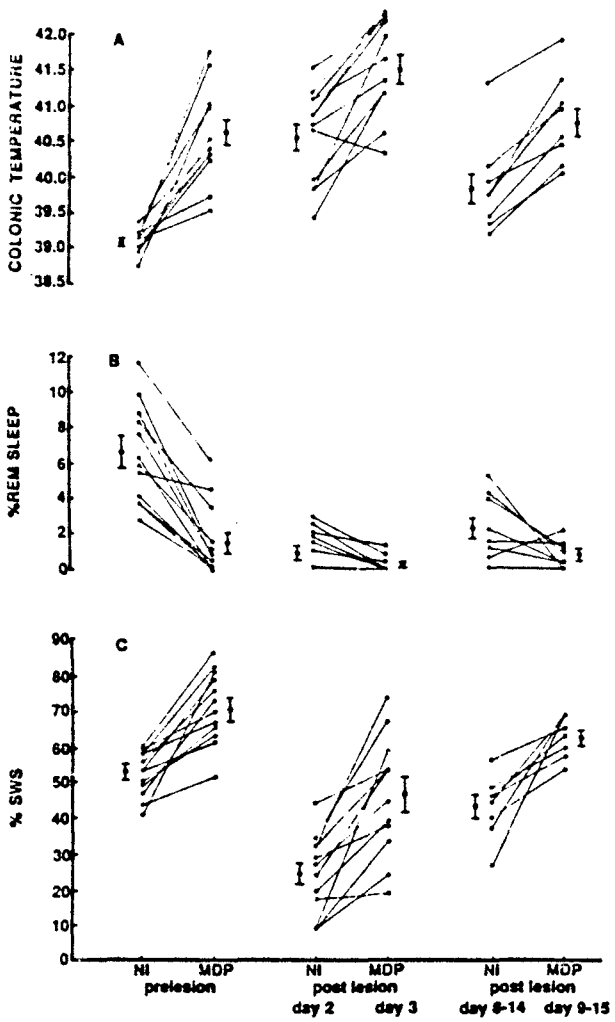


Fig. 3: Effects of MDP on rabbit SWS sleep, and T_{co} before and after preoptic area lesions. For each set of lines, the values on the left were taken during control no infusion (NI) recordings, and values on the right were taken the next day after MDP treatment. Lines connect values taken from the same rabbit. Mean values from each group ± SEM are to the left and right of each set of lines; these values correspond to those shown in Table 2. In A, T_{co} were taken 6 h after recordings were begun. Sleep values are the percent of time spent in either REM sleep (B) or SWS (C). After lesions, T_{co} increased, and MDP treatment induced an even greater increase in T_{co} in every animal but one. Eight to 14 days after lesions, T_{co} values were still significantly above prelesion values. The lesion also induced decreases in SWS and REM sleep. However, lesioned animals retained their capacity to respond to MDP in that every lesioned animal showed greater duration of SWS after MDP treatment, and of those animals that had REM sleep, it was inhibited by MDP.

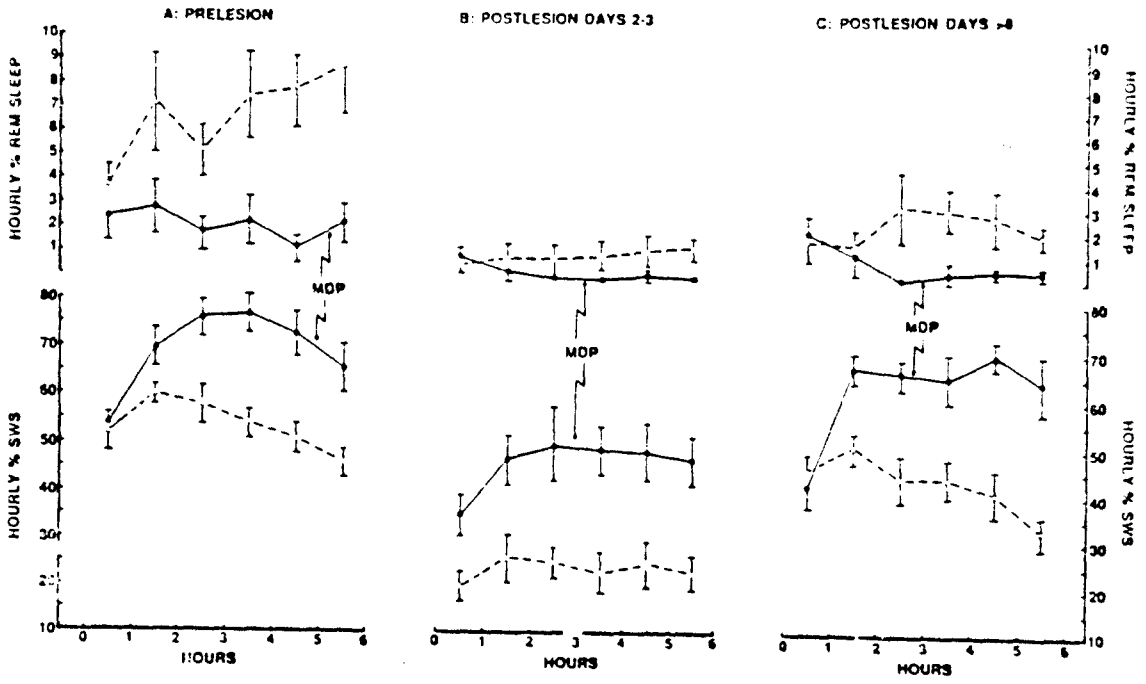


Fig. 4: Time courses of SWS and REM sleep after MDP treatment before and after lesion of the preoptic area (POA). Before (A) and after (B, C) POA lesions, the time courses of sleep responses to MDP were similar, although the baseline levels upon which responses were superimposed shifted to lower values after the lesions.

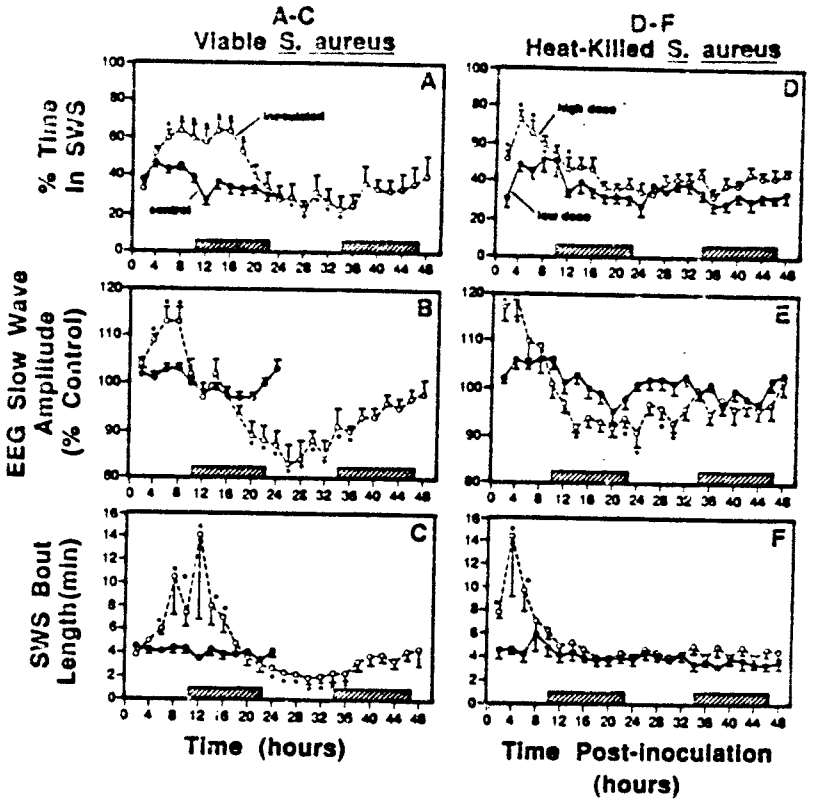


Fig. 5: Effect of inoculation with viable and heat-killed S. aureus on SWS. (Left) Panels indicate the percentage of time in SWS (a), EEG slow wave amplitude during sleep (b), and the length of individual bouts of SWS (c) of rabbits (n = 16) for 24 hr prior to (●—●) and for 48 hr after (○---○) the i.v. administration of 10^7 to 10^8 CFU of viable S. aureus. (Right) Panels indicate the percentage of time in SWS (d), EEG slow wave amplitude during sleep (e), and the length of individual bouts of SWS (f) of rabbits for 48 hr after the i.v. administration of 8 x 10^7 (●—● ; n = 8) or 7 x 10^9 (○---○; n = 12) CFU of heat-killed S. aureus. Baseline data for these animals are not shown, but were not significantly different from that presented in the panels to the left. For all panels, data points represent the mean ± S.E.M. of values obtained from each rabbit during the preceding 2-hr period. Shaded areas on the abscissa indicate the "lights-off" period. *$p < 0.03$, relative to corresponding baseline values.

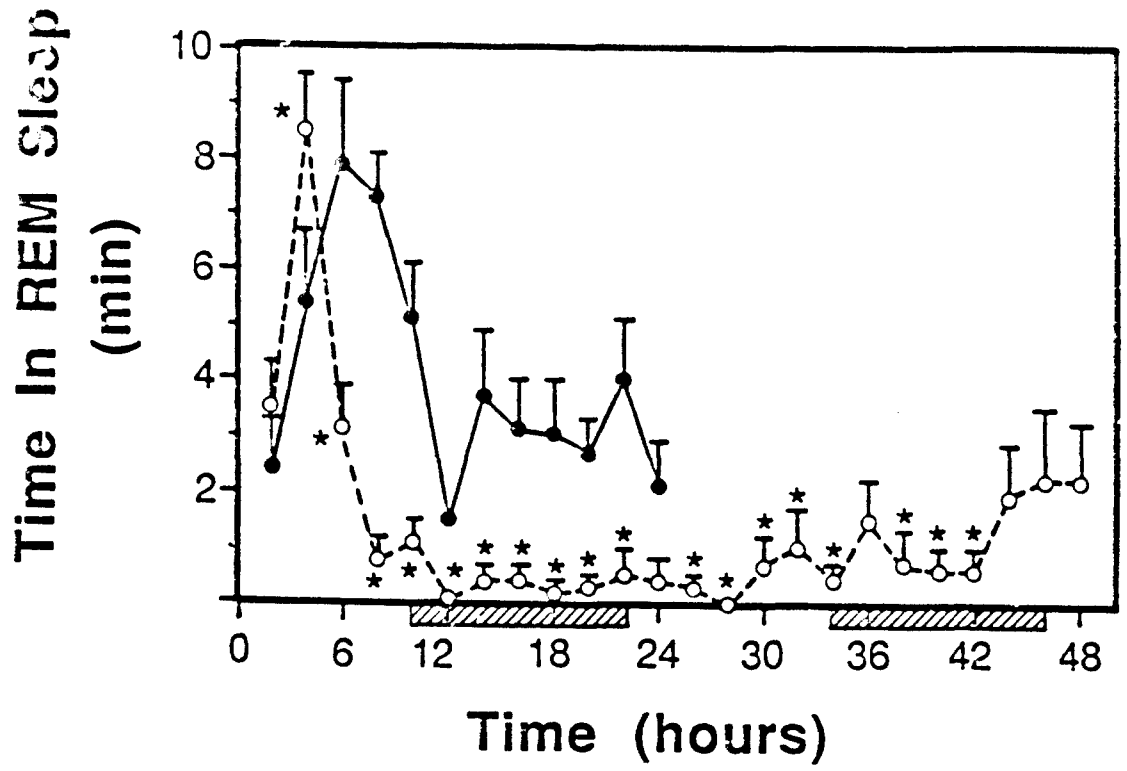


Figure 6: Effects of inoculation with viable S. aureus on rapid eye movement sleep (REMS). Total time spent in REMS during each 2-hr interval for 24 hr prior to (●—●) and 48 hr after (o---o) the i.v. inoculation of rabbits (n = 12) with 10^7 to 10^8 CFU of viable S. aureus. Data points represent the mean ± S.E.M. of values obtained from each rabbit during the preceding 2-hr period. Shaded areas on the abscissa indicate the "lights-off" period. *p < 0.01, relative to corresponding baseline values.

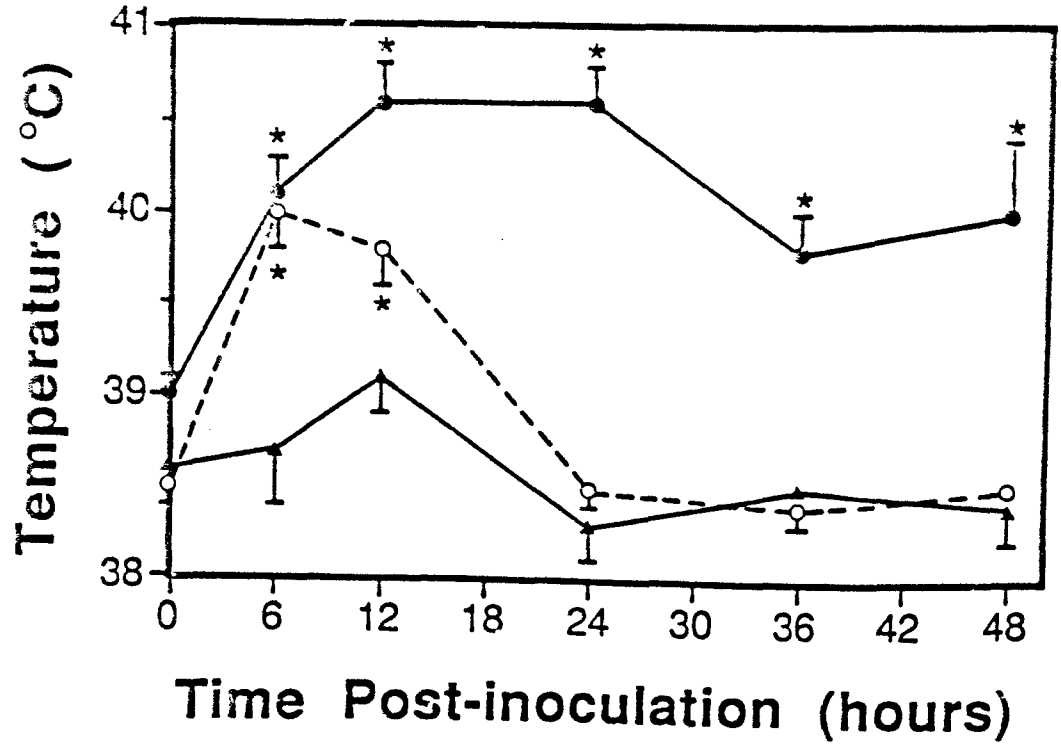


Figure 7: Effects of inoculation with viable and heat-killed S. aureus T_{co}. Colonic temperature was measured prior to and every 6-12 hr after the i.v. inoculation of rabbits with 10^7 to 10^8 CFU of viable S. aureus (●—●); n = 16), 8 x 10^7 CFU of heat-killed S. aureus (▲—▲); n = 8), or 7 x 10^9 CFU of heat-killed S. aureus (○---○; n = 12). Data points represent the mean ± S.E.M. *$p < 0.03$, relative to time 0.

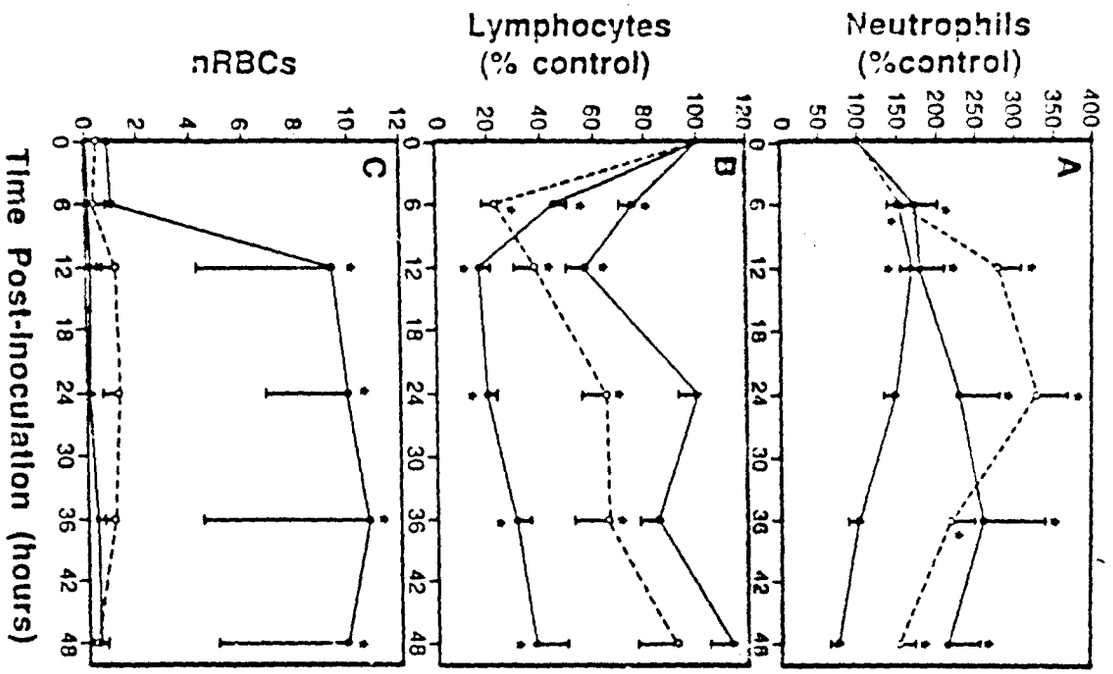


Figure 8: Effects of inoculation with viable or heat-killed S. aureus on hematological parameters. The numbers of neutrophils (a), lympocytes (b), and nRBCs (c) were determined prior to and every 6-12 h after the i.v. inoculation of rabbits with 10^7 to 10^8 CFU of viable S. aureus (●—●; n = 16), 8×10^7 CFU of heat-killed S. aureus (▲—▲; n = 8), or 7×10^9 CFU of heat-killed S. aureus (○---○; n = 12). Data points represent the mean ± S.E.M. The numbers of neutrophils and lymphocytes measured in the preinoculation period were 2557 ± 147 and 5629 ± 348 per ml of blood, respectively (n = 36). nRBCs represent the number counted per 100 WBCs. *$p < 0.03$, relative to time 0.

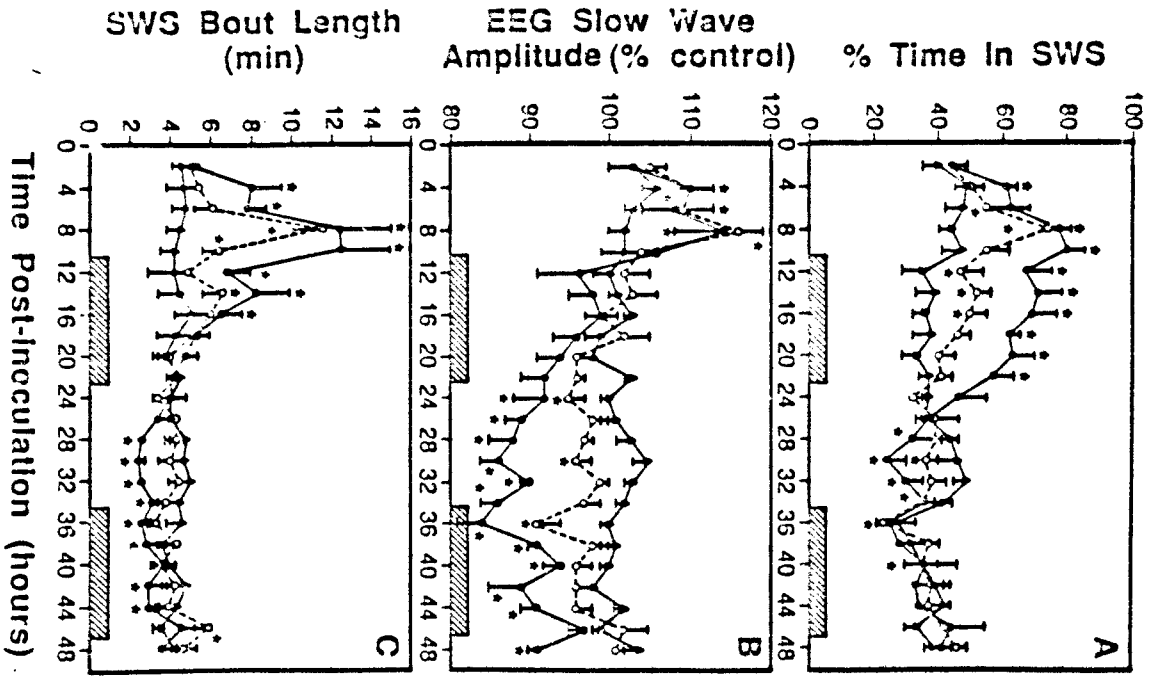


Figure 9: Effect of cephalothin on S. aureus-induced changes in sleep. Rabbits inoculated i.v. with 10^7 to 10^8 CFU of viable S. aureus also received an i.m. injection of the antibiotic cephalothin (40 mg/kg; o--o; n = 8) or the appropriate volume of saline vehicle (●—●; n = 8), both at the time of S. aureus inoculation and every 12 h thereafter. Additional animals (▲--▲; n = 6) received cephalothin injections without S. aureus inoculation. Panels indicate the percentage of time in SWS (a), EEG slow wave amplitude during sleep (b), and the length of individual bouts of SWS (c) of rabbits for 48 hr inoculation. Baseline data for these animals are not shown, but were not significantly different from those presented in Fig. 5. For all panels, data points represent the mean ± S.E.M. of values obtained from each rabbit during the preceding 2-hr period. Shaded areas on the abscissa indicate the "lights-off" period. *$p < 0.03$, relative to corresponding baseline values.

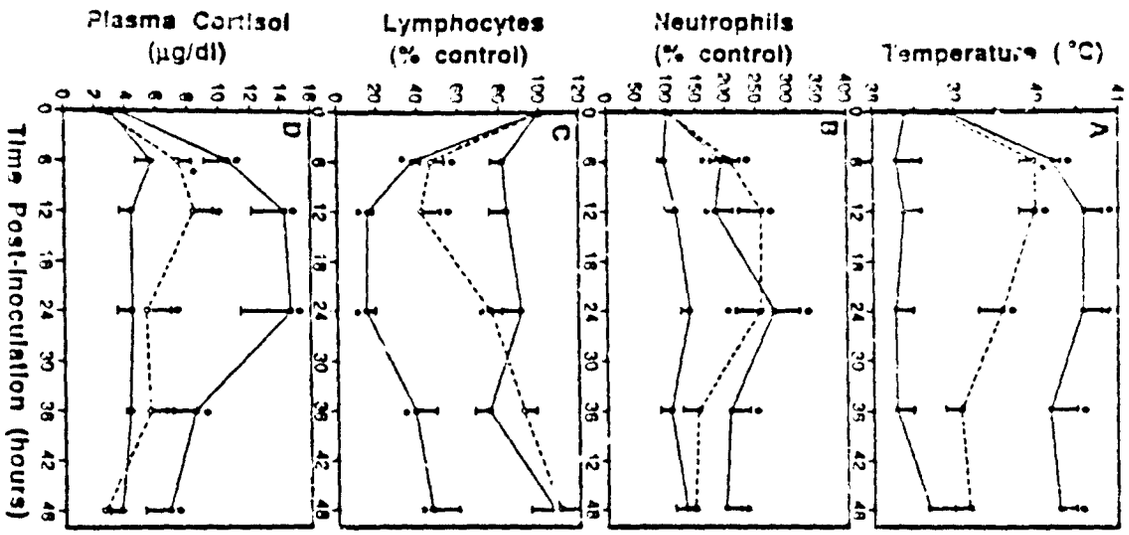
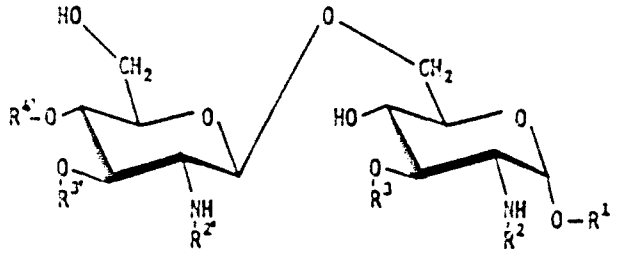


Figure 10: Effects of cephalothin on S. aureus-induced changes in temperature and hematological parameters. Rabbits inoculated i.v. with 10^7 to 10^8 CFU of viable S. aureus also received an i.m. injection of the antibiotic cephalothin (40 mg/kg; o--o ; n = 8) or the appropriate volume of saline vehicle (●—● ; n = 8), both at the time of S. aureus inoculation and every 12 hr thereafter. Additional animals (▲—▲ ; n = 6) received cephalothin injections without S. aureus inoculation. The colonic temperature (a), the numbers of neutrophils (b) and lymphocytes (c), and plasma cortisol levels (d) were measured prior to and every 6-12 hr after inoculation. Data points represent the mean ± S.E.M. The numbers of neutrophils and lymphocytes measured in the pre-inoculation period were 3065 ± 391 and 5547 ± 244 per ml of blood, respectively (n = 22). *$p < 0.03$, relative to time 0.



Compound	$R^{3'}$	$R^{2'}$	R^3	R^2	$R^{4'}$	R^1
LA-15-PP (506)	C_{14}-O-(C_{14})	C_{14}-O-(C_{12})	C_{14}-OH	C_{14}-OH	P	P
LA-15-PH (504)	C_{14}-O-(C_{14})	C_{14}-O-(C_{12})	C_{14}-OH	C_{14}-OH	P	H
LA-16-PH (514)	C_{14}-O-(C_{14})	C_{14}-O-(C_{12})	C_{14}-OH	C_{14}-O-(C_{16})	P	H
LA-18-PP	C_{14}	C_{14}-OH	C_{14}	C_{14}-OH	P	P

Abbreviations:
P= $PO(OH)_2$; C_{14}= tetradecanoyl;
C_{14}-OH= (R)-3-hyroxytetradecanoyl;
C_{14}-O-(C_{12})= (R)-3-dodecanoyloxytetradecanoyl;
C_{14}-O-(C_{14})= (R)-3-tetradecanoyloxytetradecanoyl;
C_{14}-O-(C_{16})= (R)-3-hexadecanoyloxytetradecanoyl.

Figure 11: Molecular structure diagrams of lipid-A molecules LA-15-PP, LA-15-PH, LA-16-PH, LA-18-PP, and Lipid-X.

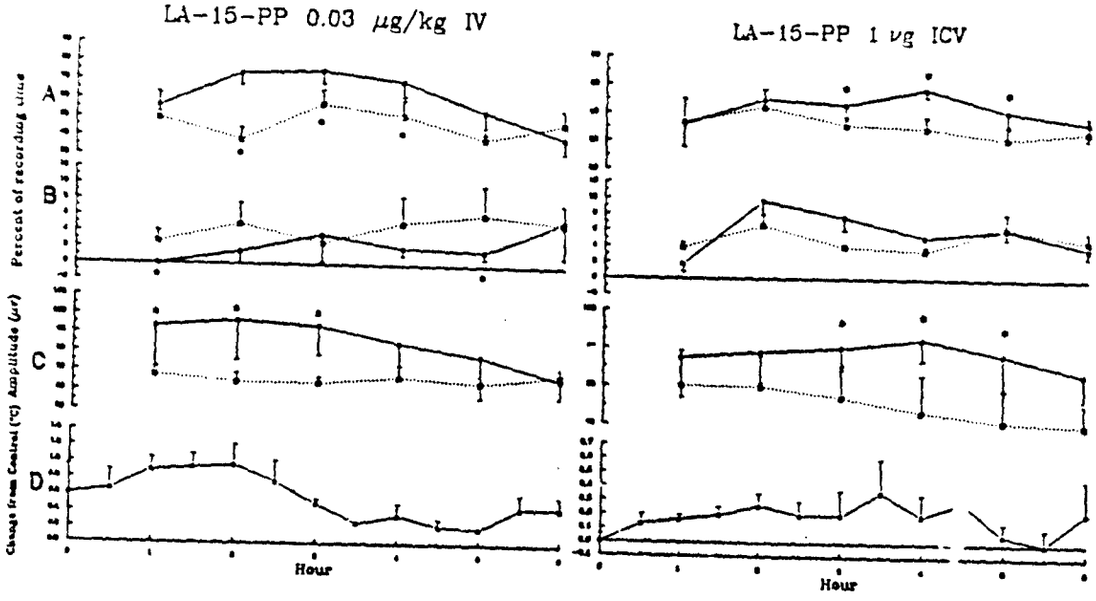


Figure 12: Effects of lipid-A analog LA-15-PP. Time courses of (A) SWS, (B) REM sleep, (C) delta EEG amplitude, and (D) brain temperature for intravenous (IV) injection (0.03 ug/kg) and intracerebroventricular (ICV) injection (1.0 ng) of lipid-A analog LA-15-PP. Symbols for sleep ad amplitudes are contrcl (■·····■) and experimental (●—●); brain temperature plot represents experimental minus control. n = 4 for all data points; *= $p < 0.05$ Wilcoxon Matched-Pairs test.

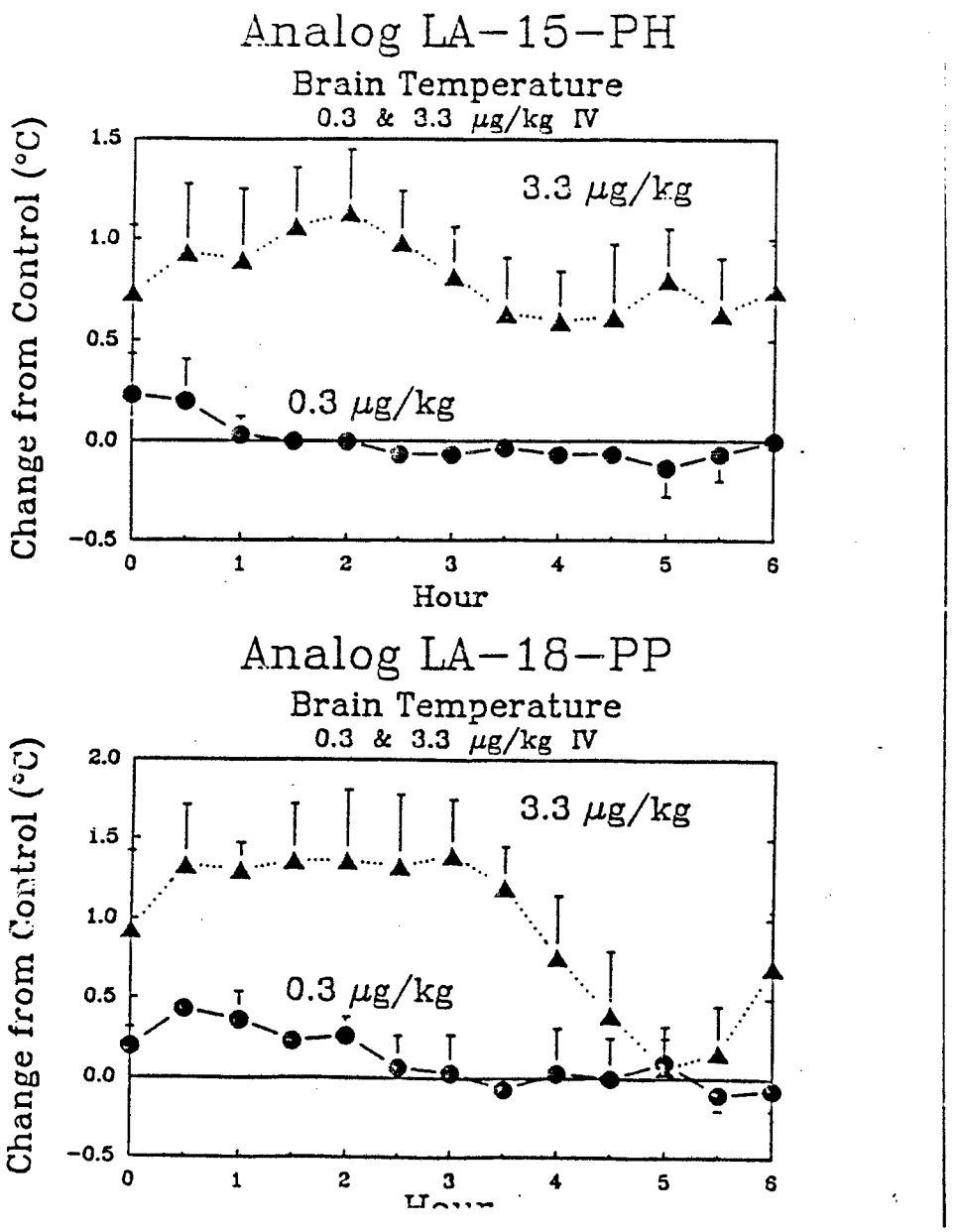


Figure 13: Course of brain temperatures after intravenous injection of lipid-A analog LA-15-PH (0.3 ug/kg; n = 4 and 3.3 ug/kg; n = 8) (top) and after intravenous injection of lipid-A analog LA-18-PP (0.3 ug/kg and 3.3 ug/kg) (bottom). The higher dose of both substances enhanced tbr whereas the lower doses were ineffective. n = 4 for all data points.

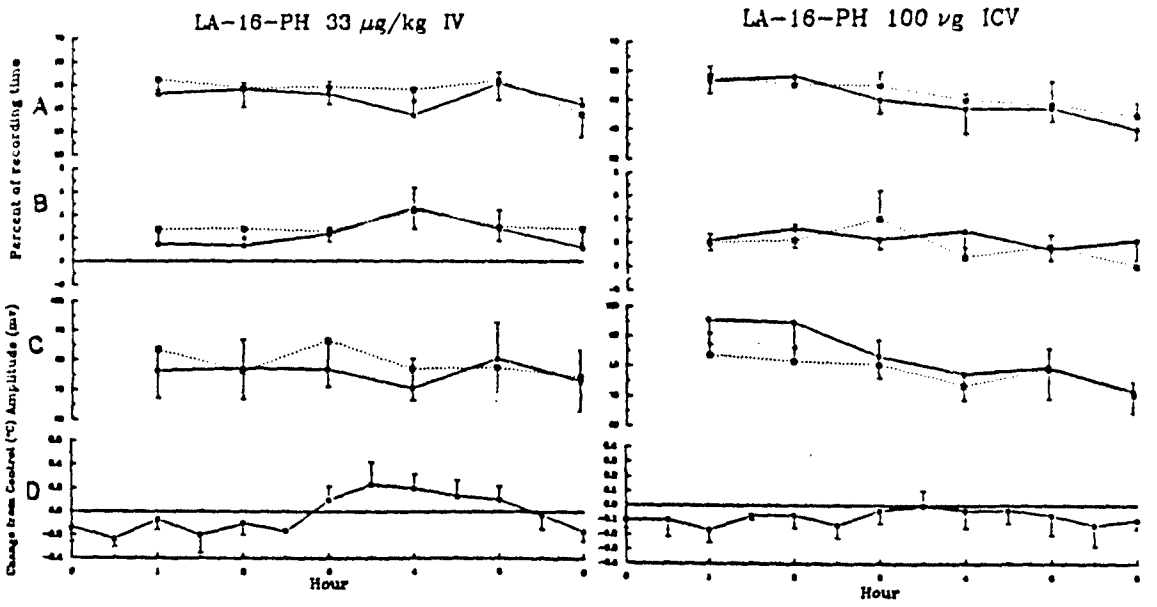


Figure 14: Effects of lipid-A analog LA-16-PH. Time courses of (A) SWS, (B) REM sleep, (C) delta EEG amplitude, and (D) brain temperature for IV injection (33.0 ug/kg) and ICV injection (100 ng) of lipid-A analog LA-16-PH. Symbols for sleep and amplitudes are control (▪···▪) and experimental (●—●); brain temperature plot represents experimental minus control. n = 4 for all data points; *p < 0.05 Wilcoxon Matched-Pairs test.

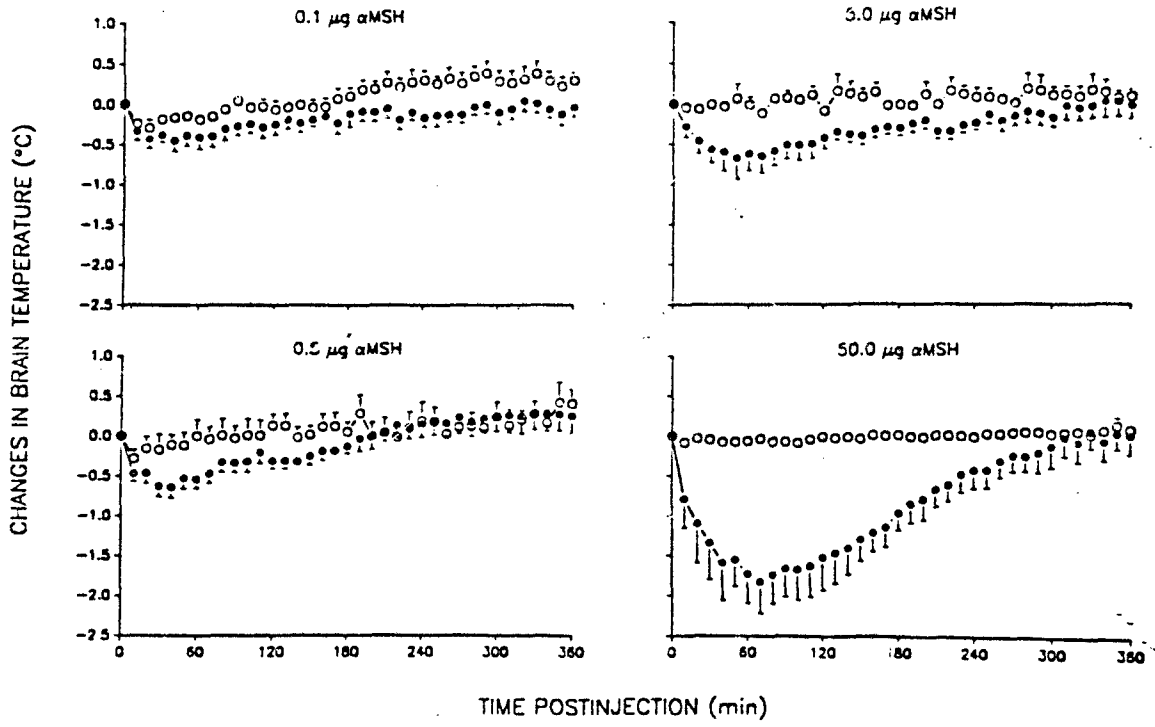


Figure 15: Time courses of brain temperature (T_{br}; °C) expressed as change from initial T_{br} after ICV injection of αMSH (solid circles) or aCSF (open circles). Values are the means (± SEM) for the following sample sizes: 0.1 ug αMSH, n = 8; 0.5 ug αMSH, n = 6; 5.0 ug αMSH, n = 5; 50.0 ug αMSH, n = 5.

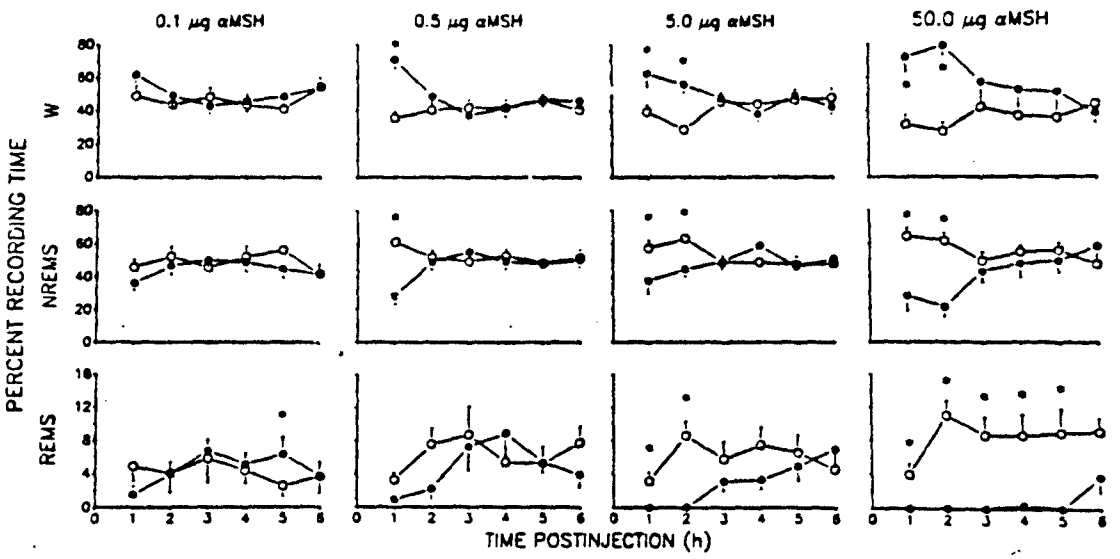


Figure 16: Effects of aMSH (solid circles) and aCSF (open circles) on percent time spent in vigilant states (W, NREMS, REMS) in postinjection hours 1-6 in rabbits. Values are means (± SEM) for eight rabbits (except 50.0 ug aMSH, n = 7). Asterisks denote significant changes from aCSF values ($P < 0.05$, Wilcoxon Matched-Pairs Signed-Ranks test).

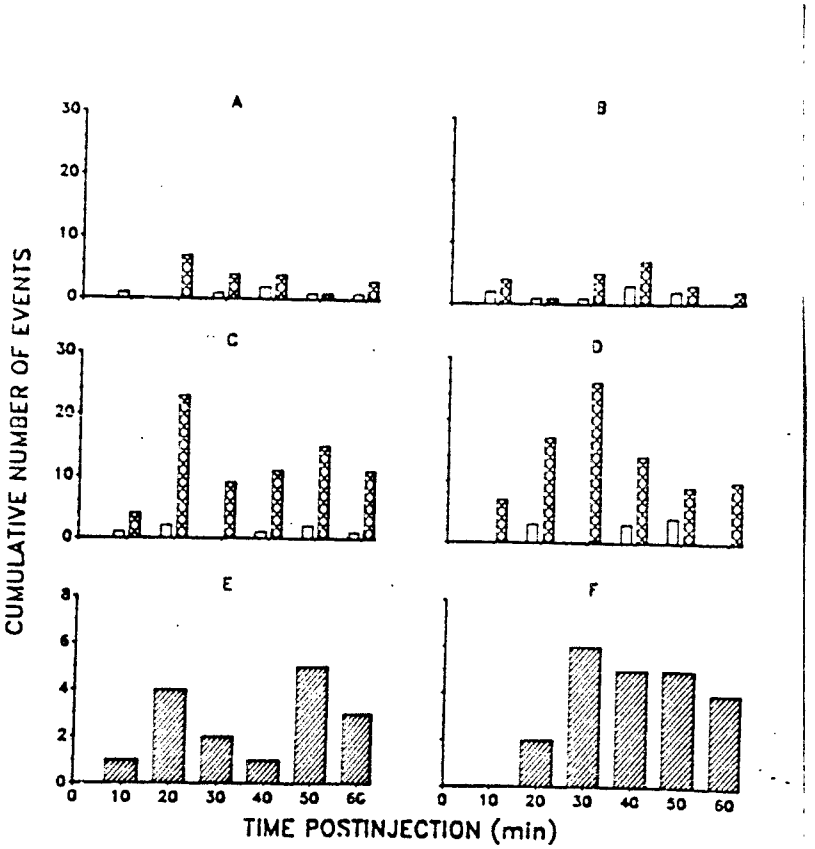


Figure 17: Cumulative number of events per 10-min time interval in postinjection hour 1 for stretching/yawning (panels A-D) and sexual excitation (panels E and F) after ICV injection of aCSF (open bars) and aMSH or IL1 + aMSH (shaded bars). Sexual excitation following aCSF injection was not observed and is therefore not depicted. A) 0.4 ug aMSH, n = 6. B) 20 ng IL1 + 0.5 ug aMSH, no = 6. C, E) 5.0 ug aMSH, n = 8. D, F) 20 ng IL1 + 5.0 ug aMSH, n = 8. For a comparison between the effects of 0.5 and 5.0 ug aMSH, see text where mean occurances of events per hour per rabbit are provided.

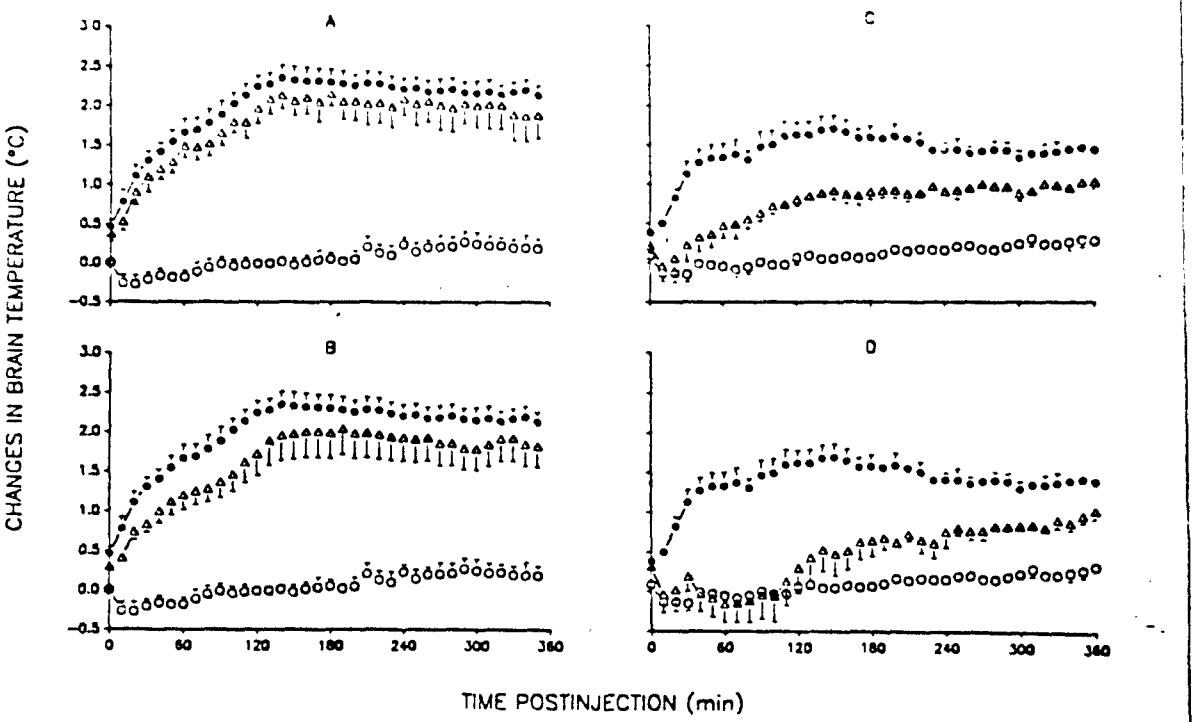


Figure 18: Mean (± SEM) brain temperature (T_{br}; °C) changes relative to initial T_{br} after aCSF (open circles), IL1 (solid circles), and IL1 + aMSH (open triangles). All injections followed a double-injection protocol (see Methods). Therefore, time 0 immediately follows the last injection. A) 40 ng IL1 + 0.1 ug aMSH, n = 7. B) 40 ng IL1 + 0.5 ug aMSH, n = 7. C) 20 ng IL1 + 0.5 ug aMSH, n = 8. D) 20 ng IL1 + 5.0 ug aMSH, n = 7.

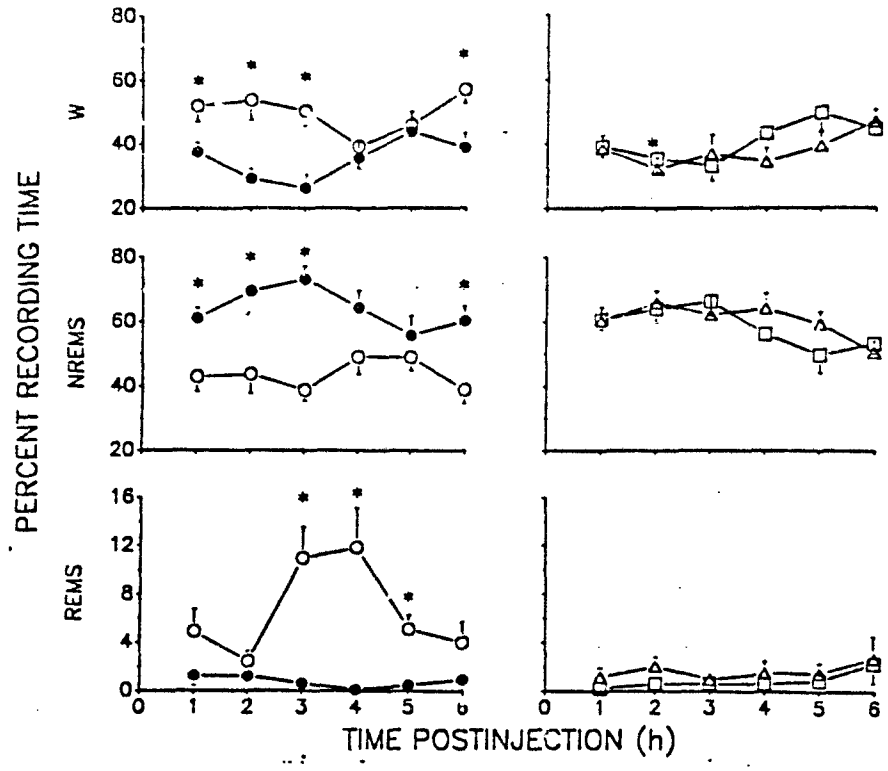


Figure 19: Effects of 40 ng IL1 and 40 ng IL1 + aMSH on rabbit sleep-wake activity. Each point represents percent time spent in vigilant states (W, NREMS, REMS) as a mean (± SEM) of eight rabbits after ICV injection of aCSF (left panels; open circles), IL1 (left panels; solid circles), IL1 ± 0.1 ug aMSH (right panels; open triangles), and IL1 + 0.5 ug aMSH (right panels; open squares). Asterisks denote significant differences ($P < 0.05$; Wilcoxon Matched-Pairs Signed-Ranks test) between aCSF and IL1 or, in the case of IL1 + aMSH doses, between IL1 and IL1 + aMSH.

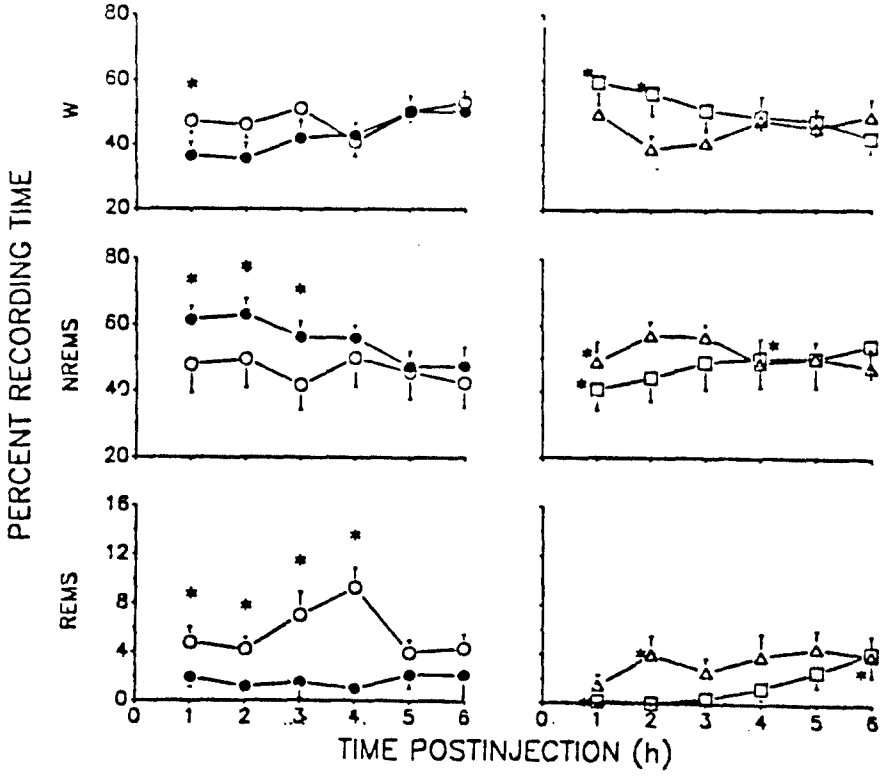


Figure 20: Effects of 20 ng IL1 and 20 ng IL1 + aMSH on rabbit sleep-wake activity. Each point represents percent time spent in vigilant states (W, NREMS, REMS) as the mean (± SEM) of eight rabbits after ICV injection of aCSF (left panels; open circles), IL1 (left panels; solid circles), IL1 + 0.5 ug aMSH (right panels; open triangles), and IL1 + 5.0 ug aMSH (right panels; open squares). Asterisks denote significant differences ($P < 0.05$; Wilcoxon Matched-Pairs Signed-Ranks test) between aCSF and IL1 or, in the case of IL1 + aMSH doses, between IL1 and IL1 + aMSH.

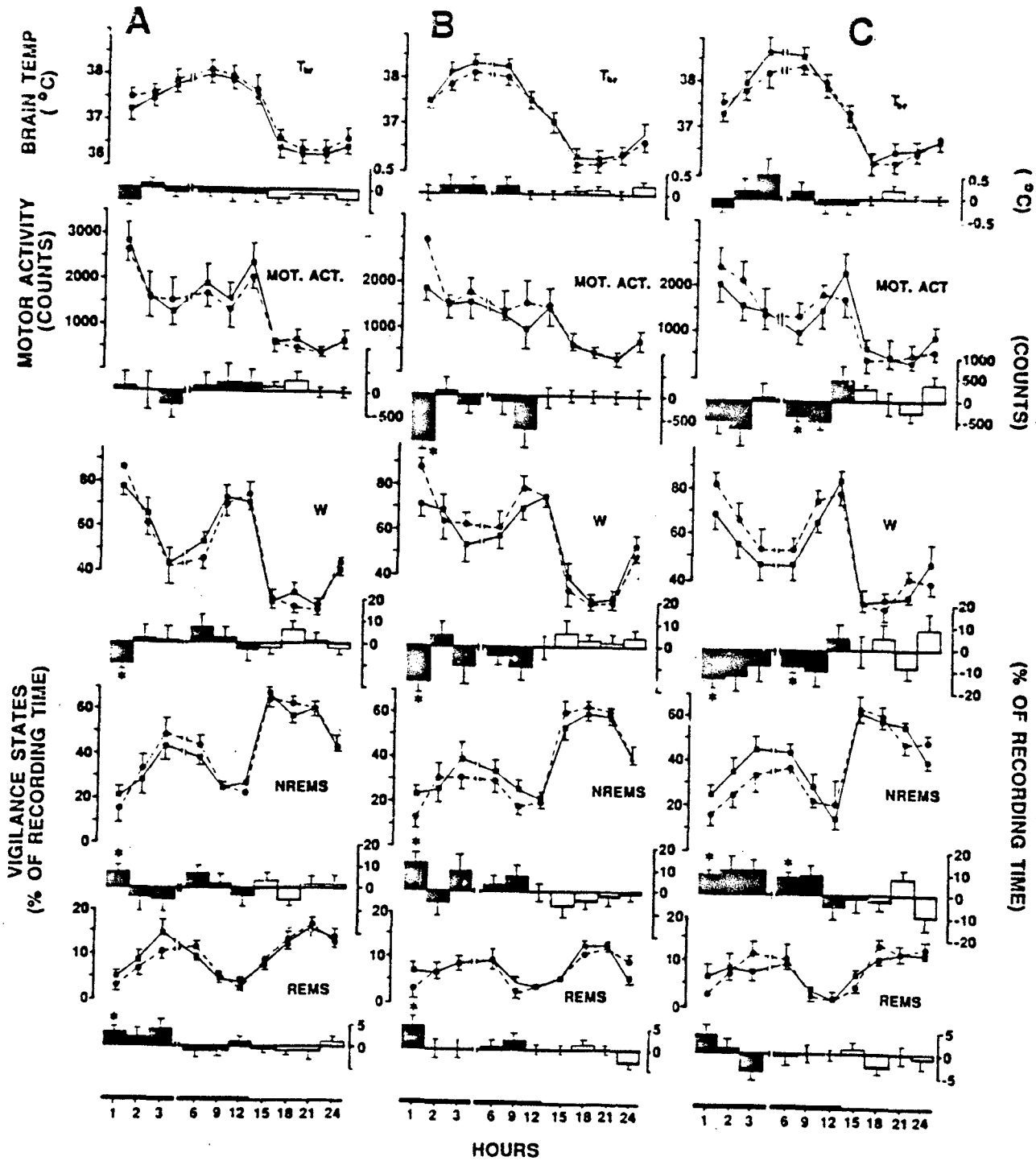

A
B
C
BRAIN TEMP (°C)
MOTOR ACTIVITY (COUNTS)
VIGILANCE STATES (% OF RECORDING TIME)
MOT. ACT.
W
NREMS
REMS
DIFFERENCE FROM BASELINE
(°C)
(COUNTS)
(% OF RECORDING TIME)
HOURS
1 2 3 6 9 12 15 18 21 24

Figure 21

Fig. 21 Effects of icv injection of GRF (column A: 0.01 nmol/kg [n = 13]; column B: 0.1 nmol/kg [n = 10]; and column C: 1.0 nmol/kg [n = 8]) on brain temperature (T_{br}), motor activity, and the percent of time spent in wakefulness (W), non-REM sleep (NREMS) and REM sleep (REMS) in rats. Courses of T_{br}, motor activity, W, NREMS and REMS are shown after icv injection of artificial cerebrospinal fluid (aCSF, broken lines) or GRF (continuous lines). Recordings were started at dark onset; the animals were injected immediately preceding the 12-h dark period (dark: shaded areas; light: open areas). Means (± SE) calculated for 1-h periods are provided for postinjection hours 1 - 3, while the means were calculated for 3-h periods for the rest of the 24-h cycle. Histograms under each pair of curves depict the differences between the baseline values and the values obtained after GRF administration. Asterisks denote significant differences (paired Student's t-test, two-tailed; $p < 0.05$).

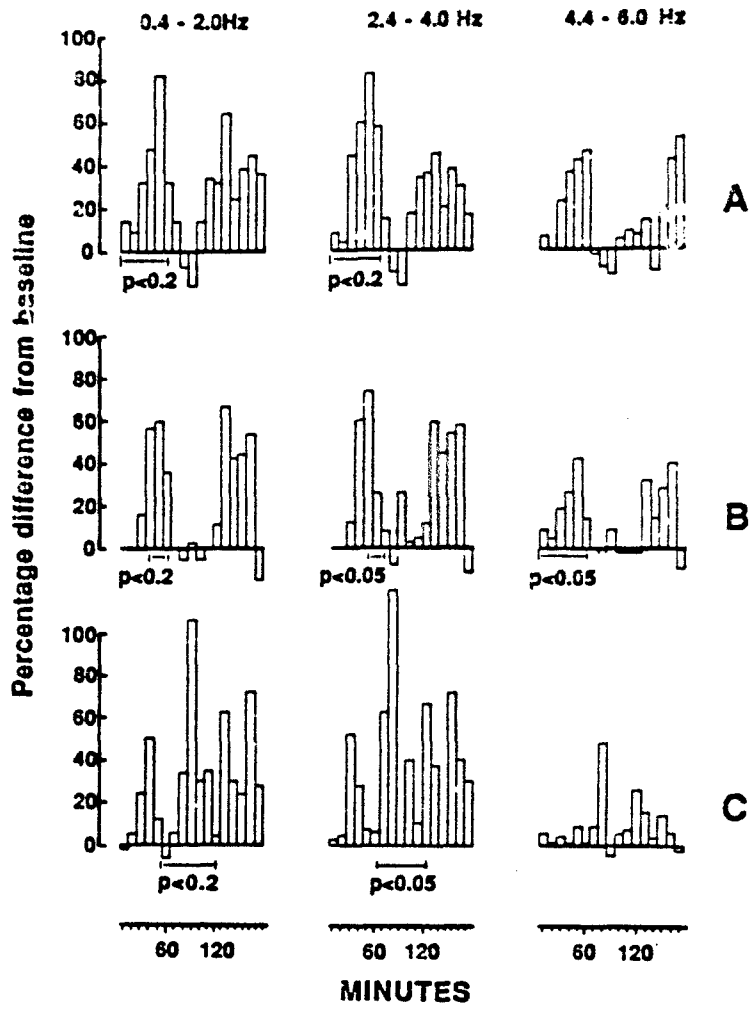


Figure 22: Changes in EEG slow wave activity following icv injection of GRF in rats. A: 0.01 nmol/kg; B: 0.1 nmol/kg; C: 1.0 nmol/kg GRF. The mean changes in power density values calculated for three frequency ranges (0.4-2.0 Hz, 2.4-4.0 Hz, 4.4-6.0 Hz) are expressed as percentage of the baseline values for 10-min periods during the first three hours after the injections. Differences between baseline values and power density values after GRF were evaluated for 30- or 60-min periods (paired Student's t-test, two-tailed).

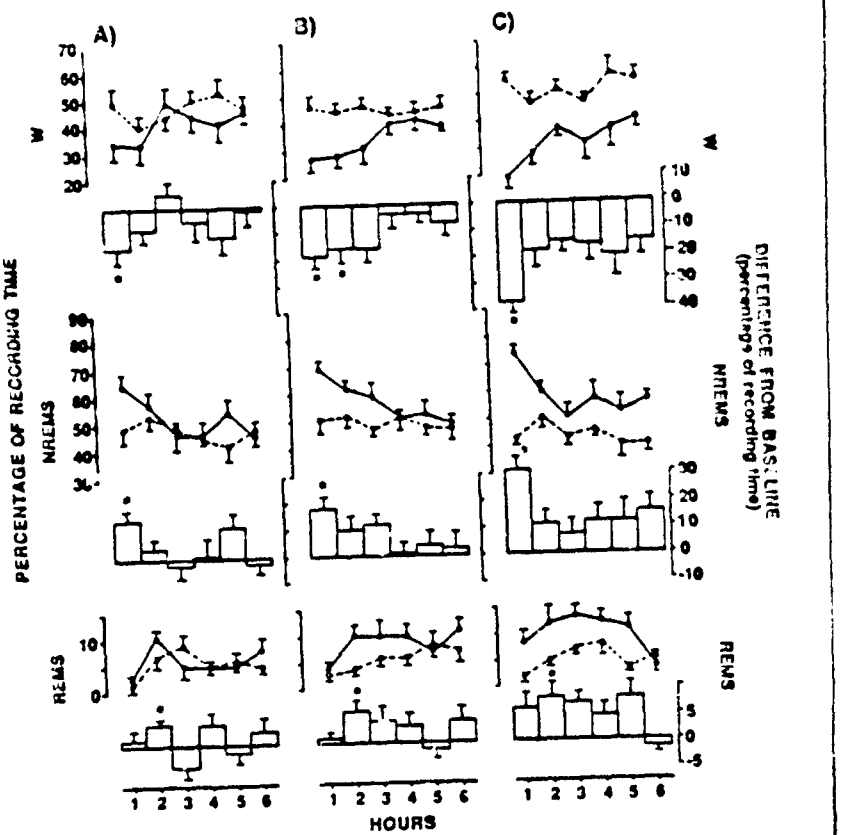


Figure 23: Effects of icv injection of GRF (column A: 0.01 nmol/kg [n = 7]; column B: 0.1 nmol/kg [n = 8]; column C: 1.0 nmol/kg [n = 6]) on sleep-wake activity in rabbits. Mean percentages (± SE) of the vigilance states (W, NREMS and REMS) are shown for each postinjection hour following icv administration of aCSF (broken lines) and GRF (continuous lines). Histograms show the mean differences (± SE) between the baseline and test values. Asterisks denote significant differences (Wilcoxon Matched-Pairs Signed-Ranks test; $p < 0.05$).

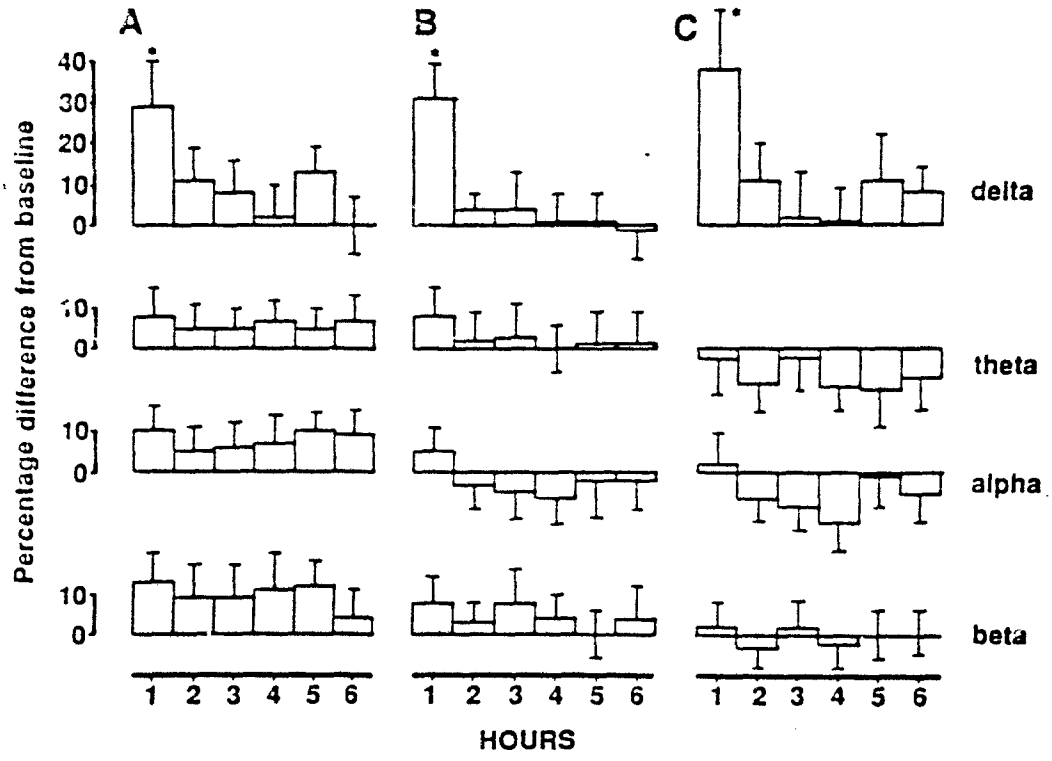


Figure 24: Changes in the mean amplitude of the EEG in four frequency bands (delta: 0.5-3 Hz; theta: 4-7.5 Hz; alpha: 8-12.5 Hz; beta: 13-25 Hz) following icv injection of GRF (A: 0.01 nmol/kg; B: 0.1 nmol/kg; C: 1.0 nmol/kg GRF) in rabbits. Changes in the amplitudes (mean ± SE) are expressed as percentages of the baseline values. Asterisks denote significant changes (Wilcoxon Matched-Pairs Signed-Ranks test; $p < 0.05$).

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